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GENETIC ANALYSIS OF THE *SACCHAROMYCES CEREVISIAE*
CENTROMERE-BINDING PROTEIN CP1

A Thesis

by

Daniel C. Masison

Submitted to the faculty of the
University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES

March

1993

To the three people without whose love and support I could not have made it this far: Allan and Patricia Masison, and my #1 CP, Cynthia Pise.

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Kevin O'Connell provided the amino-terminal truncation alleles described in Chapter V, assayed the methionine phenotypes of *cep1* strains carrying these alleles, and gave helpful advice in performing phosphatase assays.

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ABSTRACT

CP1 is a sequence specific DNA-binding protein of the yeast *Saccharomyces cerevisiae* which recognizes the highly conserved centromere DNA element I (CDEI) of yeast centromeres. The gene encoding CP1, which was designated *CEP1* for centromere protein 1, was cloned and sequenced. *CEP1* encodes a highly acidic protein of molecular weight 39,400. *CEP1* was mapped to a position 4.6 centiMorgans centromere distal to *SUP4* on the right arm of chromosome X. Phenotypic analysis of *cep1* mutants demonstrated that yeast strains lacking CP1 are viable but have a 35% increase in cell doubling time, a ninefold increase in the rate of mitotic chromosome loss, and are methionine auxotrophs. Detailed analysis of the mitotic chromosome-loss phenotype showed that the loss is primarily due to chromosome nondisjunction (2:0 segregation). During meiosis *cep1* null mutants exhibited aberrant segregation of centromere containing plasmids, chromosome fragments, and chromosomes. The predominant missegregation event observed was precocious sister segregation. The mutants also displayed a nonrandom 20% decrease in spore viability. Missegregation of chromosomes accounted for some but not all of this decreased spore viability, the remainder of which is presumed to be related to the pleiotropic consequences of the *cep1* mutation. Together with the observed mitotic missegregation phenotype the results are interpreted as suggesting that CP1 promotes sister chromatid-kinetochore adhesion. The following conclusions are based on my mutational analysis of CP1: (1) CP1 is normally present in functional excess, (2) the C-terminal 143 amino acids are sufficient for full CP1 function in chromosome segregation and methionine metabolism, and (3) while DNA binding is apparently necessary for function, DNA binding *per se* is not

sufficient. All of the mutations which caused an observable phenotype affected both centromere function and methionine metabolism. In addition, a direct correlation was observed in the degree to which both phenotypes were affected by different mutations. None of the mutant proteins displayed trans-dominant effects in a wild type background; however, two nonfunctional DNA binding-competent mutants exerted a dominant negative effect on the ability of PHO4 to suppress *cep1* methionine auxotrophy. The data are consistent with a model in which CP1 performs a similar function at centromeres and promoters.

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CHAPTER I

INTRODUCTION

Mitosis and meiosis are processes by which replicated genomes, carried on a number of separate chromosomes, are properly distributed upon cell division. Mitosis can be divided into five cytologically defined phases which are briefly summarized here. During prophase the replicated chromosomes, which exist as pairs of identical sister chromatids that remain attached at their centromeres, condense, and a microtubule-based spindle forms between poles at opposite sides of the nucleus. At prometaphase kinetochore structures assemble at the centromeres and mediate the attachment of each chromosome to spindle microtubules originating from both poles. The chromosomes oscillate along the microtubules until they congress at a central plane in the midzone (the metaphase plate). During metaphase chromosomes become held in tension at the metaphase plate with the kinetochores of the two sister chromatids facing opposite poles. Abruptly, the sister chromatids of each chromosome, which remain attached to this point, separate and segregate to opposite sides of the cell by moving poleward along the microtubules to which they are attached (anaphase A) and by separation of the spindle poles (anaphase B). At telophase, each group of segregated chromosomes becomes enclosed in a separate nuclear membrane and the cell soon divides resulting in two genetically identical daughter cells.

In meiosis the genome of a diploid cell is replicated and then undergoes two successive divisions resulting in the production of four haploid gametes. Although the mechanisms involved in chromosome segregation in meiosis are similar to those in mitosis, there is a fundamental difference in that the separation of sister chromatids is delayed until the second meiotic division. During the first meiotic division (MI) homologous chromosomes

become paired with each other and the kinetochores of each homolog attach to spindle microtubules originating from a single pole rather than from both poles. At anaphase, homologous chromosomes segregate to opposite poles while the sister chromatids of each homolog remain attached at the centromere and segregate together. During the subsequent division (MII) the sister chromatids separate and segregate in a manner resembling mitosis.

In order for chromosomes to be segregated accurately during these processes, mechanisms must exist which ensure that sister chromatids remain attached until anaphase, that the chromosomes associate with the spindle microtubules in a manner which results in their proper alignment prior to anaphase, and which provide a force for poleward movement of the chromosomes during anaphase. The functional importance of the kinetochore in each of these mechanisms has become evident (103). Although historically the terms kinetochore and centromere have been used interchangeably to define the site on the chromosome where the spindle microtubules attach, a distinction between these two elements has come to be generally accepted. The centromere consists of the DNA sequence on the chromosome upon which the kinetochore assembles and which serves as the site where the adhesion of the sister chromatids is maintained until they are due to be separated. The kinetochore is a proteinaceous cellular organelle that acts as an interface between the centromeric DNA and the spindle microtubules (13).

Kinetochores have been shown to be capable of capturing and stabilizing microtubules which originate from the spindle poles (42, 66) and have been suggested as being responsible directing the oscillatory motions of chromosomes during prometaphase (6, 86). More recently, evidence has been

obtained which suggests that kinetochores possess motors for both poleward chromosome movement (74, 84, 105) and movement of chromosomes away from the pole (49). Furthermore, specific kinetochore proteins have been implicated in generating the forces which drive chromosome movement during anaphase A (48, 49, 106). Together these results suggest that the kinetochore is primarily responsible for the proper alignment and movement of chromosomes in mitosis. It has also been suggested that kinetochore proteins play a role in the mechanism for maintaining sister chromatid attachment (81, 83). Although one view for this mechanism is that the replication of centromere DNA is delayed until anaphase (2), in *Saccharomyces cerevisiae* this possibility has been ruled out by experiments which demonstrated that centromere DNA is replicated prior to the onset of mitosis (62).

The study of centromeres of human chromosomes revealed that they consist of tandem arrays of repeated sequence elements, dominated by 170 bp units known as α -satellite DNA, that range in length from 300-5000 kb (reviewed in (104)). The centromeric regions of human metaphase chromosomes have atypical chromatin structures due to the preclusion of the final stages of chromatin condensation (82). The resulting chromatin, which has a reduced diameter and is more extended in comparison with the chromosome arms, is defined cytologically by a large narrowly constricted area known as the primary constriction (82). The kinetochores assemble on the lateral edges of this primary constriction forming trilaminar structures to which a dozen or so spindle microtubules attach (103).

The discovery of autoantibodies directed against centromere proteins in CREST scleroderma patients (67) has facilitated the study of human kine-

chore proteins. Although several proteins associated with human centromeres (termed CENPs for centromere proteins) have been identified, the specific roles that these proteins play in kinetochore function have yet to be determined. Among the partially characterized CENPs are CENP-A, a centromere specific histone-like protein (76), and CENP-B, a highly acidic b-HLH DNA-binding protein which binds to sites contained within the α -satellite DNA repeats (93). One or both of these proteins may contribute to the atypical chromatin structure of the centromere. A third human kinetochore protein, CENP-E, has been cloned, sequenced, and shown to be highly homologous to the kinesin family of microtubule motor proteins (106). Immunocytological assays show that this protein accumulates just before mitosis, associates with kinetochores during chromosome congression, relocates to the spindle mid-zone at anaphase, and is rapidly degraded at the end of cell division. Although functional assays of CENP-E have yet to be done, the circumstantial evidence suggests that CENP-E is responsible for chromosome movement during anaphase A and/or spindle elongation during anaphase B (106).

The centromeres of the three chromosomes of the fission yeast *Schizosaccharomyces pombe* are structurally similar to mammalian centromeres in that they also contain regions of repetitive DNA sequences. However, the overall length of these centromeres and the number of repeats are smaller. The length of the centromere from chromosome 1, the simplest *S. pombe* centromere, spans about 40 kb and contains a central core of 4-5 kb flanked by a 34 kb repeat (39). These flanking repeats both contain three repeated elements. The other two *S. pombe* centromeres are slightly more complex in that they contain additional copies of these same repeats, or in ad-

dition contain copies of different repeated sequences that are specific to the particular centromere (20). Although the central cores of the three centromeres do not cross hybridize (21), it is possible that small conserved sequence elements exist within these regions. Deletion analysis of the repeated elements of these centromeres revealed that certain of the conserved repeats are important for maintaining sister chromatid attachment during MI while showing little functional importance during either MII or mitosis (21).

Although no *S. pombe* kinetochore proteins have been identified to date, and it has yet to be addressed directly, it is likely that sites for centromere-specific DNA binding proteins are contained within these repeats.

As a model system, *Saccharomyces cerevisiae* (hereafter referred to as yeast) provides several advantages for studying centromere function. The centromeres of *S. cerevisiae* chromosomes are simpler still and span only about 125 nucleotides. Mitotic chromosome segregation in yeast occurs with very high fidelity; the loss rate for a given chromosome is only one to two events in every 10^5 cell divisions (41). When in the diploid state and under appropriate environmental conditions, yeast cells are capable of undergoing meiosis and sporulation which results in the formation of four haploid derivatives from a single diploid cell. Chromosome segregation during meiosis also occurs with high fidelity; aberrant segregation of a given chromosome occurs only once in 10^4 meioses (91). Well developed genetic methods exist for monitoring chromosome segregation in yeast during both mitosis and meiosis (55, 90). Additionally, a relatively high level of aneuploidy can be tolerated in this organism without a loss of viability or a significant change in growth rate (79).

Although, like those of *S. pombe*, *S. cerevisiae* centromeres do not cross hybridize, eleven of this organism's sixteen centromeres have been sequenced and they show a high degree of conservation (reviewed in (32)). The centromere is made up of three distinct and highly conserved sequence elements termed CDEI, CDEII, and CDEIII (33, 44). Together, these elements comprise the functional centromere and when used to replace the resident centromere of a yeast chromosome are sufficient to provide full mitotic and meiotic centromere function (22, 72). CDEI is the degenerate octanucleotide RTCACRTG (R=purine), CDEIII is a partially palindromic sequence 25 bp in length, and CDEII is a 78-86 bp region of highly A+T-rich (>90%) DNA which seems to provide a spacer function (32). Mutational analyses have revealed that CDEIII is absolutely essential for mitotic centromere function (43, 63, 77), while mutations of CDEI and CDEII impair but do not abolish function (23, 35, 43, 77).

The structure of the yeast kinetochore also appears to be relatively simple. Electron micrographs of mitotic yeast nuclei fail to reveal an obvious kinetochore structure: spindle microtubules seem to attach directly to the chromatin fibers, at a ratio of one microtubule per centromere (80). However, nuclease sensitivity studies of native yeast chromatin show that the centromeric DNA is highly resistant to digestion and is flanked on both sides by nuclease hypersensitive sites associated with highly phased nucleosome arrays. These observations have led to speculation that the nuclease resistant core represents a structurally primitive kinetochore (8). Two centromere-specific DNA binding proteins have been identified, one which binds to CDEI and one which binds to CDEIII. The CDEIII binding factor, CBF3, is actually a

complex of three different proteins, at least one of which is phosphorylated (58). Hyman *et al.* recently demonstrated that CBF3 possesses minus-end directed microtubule-motor activity (48). This study led to the suggestions that the motor activity of CBF3 is responsible for the anaphase A movement of yeast chromosomes along microtubules and that microtubule-based motors are an essential part of the DNA-microtubule linkage.

CP1 is a relatively abundant yeast protein that binds to CDEI (3, 11, 16, 52). Hegemann *et al.* tested the effect of several point mutations of CDEI on the mitotic stability of 150 kb chromosome fragments and found 2- to 10-fold elevated loss rates depending on the mutation (43). Deletion of CDEI results in a 10- to 60-fold increase in the rate of mitotic chromosome loss (23, 36, 77) and in an increase in the rate of missegregation of a centromere-containing plasmid during meiosis (23, 36). A correlation has been made between the avidity of the DNA binding by CP1 at centromeric CDEI sites and mitotic chromosome stability (3), implicating CP1 as a functional component of the *S. cerevisiae* kinetochore.

CEP1 (also known as *CBF1* and *CPF1*), the gene encoding CP1, has been cloned and sequenced (5, 17, 64). *CEP1* encodes a 351 amino acid polypeptide with a deduced molecular weight of 39,400 that is a member of the basic-helix-loop-helix (b-HLH) family of DNA binding proteins (5, 17). The molecular weight of CP1 determined by SDS gel electrophoresis is 58,000 (3), significantly larger than its predicted molecular weight. However, when expressed in *E. coli*, *CEP1* directs the synthesis of a CDEI sequence-specific DNA binding protein with an SDS gel mobility similar to that of CP1 purified from yeast, indicating that CP1 migrates abnormally in SDS gels (5). A sporulated diploid

strain carrying a null mutation in *CEP1* (*[cep1::URA3]-11*) gives rise to asci containing four viable spores which segregated the *URA3* marker 2⁺:2⁻, indicating that *CEP1* is not an essential gene and also that it is present in single copy in the yeast genome (5). The *[cep1::URA3]-11* segregants do not possess CDEI specific DNA binding activity, indicating that the disruption results in the loss of functional CP1 protein from the cell (5).

Not surprisingly, yeast strains lacking CP1 exhibit increased rates of chromosome loss. The *[cep1::URA3]-11* disruption causes a ninefold increase in the rate of the mitotic loss of chromosome *III* from $1-2 \times 10^{-5}$ to $2-3 \times 10^{-4}$ events per cell per division (5), quantitatively similar to that observed for a chromosome with a CDEI deletion in a wild type strain (36). Additionally, measurements of the mitotic loss rates of plasmids containing intact and CDEI deleted centromeres for wild type and *cep1* strains show that the *cis* effect of deleting CDEI from the centromere and the *trans* effect of removing CP1 from the cell are equivalent and non-additive. Taken together, these results demonstrate that the involvement of CDEI in mitotic centromere function is mediated through an interaction with CP1 (5).

Interestingly, mutant strains which lack functional CP1 display two additional phenotypes. First, it is immediately obvious that *cep1* strains grow more slowly than their isogenic *CEP1* counterparts. When quantitated, both *cep1* haploids and homozygous *cep1* diploids are shown to have a 35% increase in generation time compared with wild type strains (5). Second, strains lacking CP1 are unable to grow in media lacking methionine. This phenotype is genetically linked to the *[cep1::URA3]-11* disruption indicating that CP1 is required for methionine prototrophy (5). Finally, all of the observed pheno-

types associated with the [*cep1::URA3*]-11 disruption are recessive in that *cep1/CEP1* diploids and *cep1* strains carrying *CEP1* on a single copy plasmid are phenotypically indistinguishable from wild type strains (5).

The overall goal of the research described by this thesis was to obtain a better understanding of the molecular mechanisms involved in chromosome segregation. The research focused specifically on the *Saccharomyces cerevisiae* centromere-binding protein CP1. The first step I took in the genetic analysis of CP1 was to isolate the gene encoding CP1 which we designate *CEP1* (for centromere protein 1). This allowed the construction and phenotypic analysis of yeast strains in which *CEP1* was disrupted. The results of that analysis, which are reviewed above, were reported in a publication entitled "Isolation of the Gene Encoding the *Saccharomyces cerevisiae* Centromere-Binding Protein CP1" (5). Since that work was carried out by several members of the Baker laboratory, it is not included in the main body of this thesis. However, since it provides important background information, the manuscript is included in its entirety as an appendix. My contributions included cloning *CEP1*, sequencing the gene, mapping the *CEP1* genomic locus, surveying for growth requirements of the *cep1* null mutant, and demonstrating the genetic linkage between the null mutation and the methionine auxotrophy. In addition to the cloning of the gene, preliminary experiments that were not included in the manuscript are described in Chapter III.

Despite the lack of CP1, *cep1* strains maintain a relatively high fidelity of mitotic chromosome transmission. However, we observed that the overall viability of spores derived from homozygous *cep1* diploids was significantly reduced. This led to the question of whether CP1 has a different or more im-

portant role in yeast meiosis. A comprehensive analysis of meiosis in *cep1* strains was undertaken to address this question. The results of that analysis, which led to a proposed role for CP1 at the centromere, are presented in Chapter IV. In order to provide additional insight into how CP1 functions in this role, a mutational analysis of CP1 was undertaken. This analysis, described in Chapter V, led to a proposed model for CP1 function in both chromosome segregation and in methionine metabolism.

CHAPTER II

MATERIALS AND METHODS

Cloning of CEPI. Purified CP1 protein (3) was subjected to trypsin digestion, and the partial amino acid sequence of two peptides (NCT14, NCT11) was determined. This analysis was performed at the Harvard Microchemistry Facility using the method described by Aebersold *et al.* (1). The peptide sequences were used to design two nondegenerate oligonucleotide probes, shown below:

NCT14

AspGlnGlyLeuLeuSerGlnGluSerAsnAspGlyAsnIleAspSerAlaLeuLeuSer
5'-GATCAAGGTTTGTGTCTCAAGAATCTAACGATGGTAACATT-
GATTCTGC-3'

NCT11 AlaIleThrProSerAsnGluGlyValLysProAsnThr[Gly/Ser]Leu
5'-GCTATTACTCCATCTAACGAAGGTGTTAAGCCAAACAC-3'

In all but one case (AAG for Lys), we chose codons predicted from yeast codon usage frequencies supplied with the DNASTAR computer software package (DNASTAR, Inc., Madison, WI). The NCT14 and NCT11 oligonucleotides were synthesized, labeled with [α - 32 P]ATP using T4 polynucleotide kinase, and used to screen a commercially available (Clontech, Palo Alto, CA) yeast genomic λ gt11 library (*Saccharomyces cerevisiae* strain X2180, ATCC #26109) by hybridization (50).

The phage library of which 23% of the phage were recombinant, contained 2 million independent clones with insert fragments ranging in size from 1.5-6 kb. 15,000 phage mixed with Y1090 host cells were plated on each

of four 150 mm plates. After growing at 42° for 6 hours, duplicate nitrocellulose filters were overlayed for 60 s on each plate. Filters were processed as described by Maniatis (60), blotted dry and then baked for 2 hours at 80° under vacuum. Filters were then hybridized overnight at 37° with the NCT14 oligonucleotide probe, washed first at room temperature for 30 min and then at 40° for 60 min, dried, and exposed to film. Two positive signals were obtained. A similar additional screen was done using 6 plates with 45,000 pfu per plate and positive signals were obtained at a frequency of 1 per 3,500 recombinant plaques. After secondary and tertiary screening, two positively hybridizing phage, λ CP1-9.1.3 and λ CP1-3.4, were selected for further use. As a final check before subcloning the insert fragments from these phages, the NCT11 oligonucleotide was tested to see if it could detect the λ CP1-3.4 phage. This phage was mixed at a ratio of 1:1 with phage from the λ gt11 library and roughly 100 pfu were plated on two 90 mm plates. One of a pair of duplicate filters from each plate was probed using the NCT11 oligonucleotide while the corresponding filters were probed using the NCT14 oligonucleotide. Both plates gave rise to 45 plaques which hybridized to both probes. Restriction endonuclease analysis indicated that the clones contained overlapping inserts. The λ CP1-9.1.3 isolate was selected as the source of the CP1 gene.

Yeast strains and media. Yeast strains used are described in Table 1. All except the chromosome *I*-marked strains (D77-R1, D92, and D93), and the R31 strains are congenic to strain 381G (40). Strain construction was carried out using standard methods (89) and in all cases involved multiple backcrosses. The *cep1::URA3* and *cep1::TRP1* alleles have been described (5, 75) (see also Figure 3). The *cep1::URA3* alleles replace deleted *CEP1* coding and 3' flanking

TABLE 1
Yeast strains

Strain	Genotype ^a
381G	<i>MATa cry1 ade2-1 trp1 his4-580 tyr1 lys2 SUP4-3</i>
D1-1C	[381G] <i>MATa leu2 ura3 ade3 TYR1 [cep1::URA3]-11</i>
D1-11D	[381G] <i>MATa leu2 ura3 ade3</i>
D1-11D.R1	[381G] <i>MATa leu2 ura3 ade3 TRP1</i> (Spontaneous <i>TRP1</i> revertant of D1-11D)
R10-2D	[381G] <i>MATa leu2 ura3 TYR1 [cep1::URA3]-10</i>
DJ115-12	[381G] <i>MATa leu2 TYR1 HIS4 cyh2 ste2-3^{ts}</i>
R26-R1	[381G] <i>MATa leu2 TYR1 HIS4 cyh2 ste2-3^{ts} can1</i> (Spontaneous <i>can1</i> mutation in DJ115-12)
D32-R1	[381G] <i>MATa leu2 ura3 ade3 TYR1 trp1::LEU2 [cep1::URA3]-10</i>
D28-6D	[381G] <i>MATa leu2 ura3 TYR1 trp1 [cep1::URA3]-10</i>
D36-R3	[381G] <i>MATa leu2 ura3 TYR1 trp1 cep1::ura3</i> (Spontaneous <i>ura3</i> mutation in strain D28-6D)
D30-18B	[381G] <i>MATa leu2 ura3 ade3 TYR1 TRP1</i>
D41.3	[381G] <i>MATa leu2 ura3 ade3 TYR1 TRP1 [CFIII(D8B.d.D30-18B)]</i>
DJ1149	[381G] <i>MATa leu1 ADE2 ade3 ade5 ade6 TRP1 HIS4 cyh2</i>
D64.5	[381G] <i>MATa leu2 ura3 ade3 TYR1 TRP1 cep1::ura3 cyh2</i> [CFIII(D8B.d.D30-18B.LEU2)]
DJ1136	[381G] <i>MATa ADE2 ade6 his3 HIS4 leu2 TYR1 cyh2</i>
D24	[381G] <i>MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1</i> <i>trp1/TRP1 [pDK243 (CEN3 LEU2 ade3-2p)]</i>
D25	[381G] <i>MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1</i> <i>trp1/TRP1 [cep1::URA3]-11/[cep1::URA3]-11 [pDK243 (CEN3</i> <i>LEU2 ade3-2p)]</i>

TABLE 1 cont'd.

D30	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1 trp1/TRP1 [cep1::URA3]-11/CEP1 [pDK243 (CEN3 LEU2 ade3-2p)]
D38	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 trp1::LEU2/TRP1 HIS4/his4 can1/CAN1 cyh2/CYH2
D39	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 trp1::LEU2/TRP1 cep1::ura3/CEP1 HIS4/his4 can1/CAN1 cyh2/CYH2
D40	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 trp1::LEU2/TRP1 cep1::ura3/cep1::ura3 HIS4/his4 can1/CAN1 cyh2/CYH2
D47	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1::LEU2 HIS4/his4 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)]
D45	[381G] MATa/MATa leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1::LEU2 cep1::ura3/cep1::ura3 HIS4/his4 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)]
D62	[381G] MATa/MATa LEU1/leu1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1::LEU2 CEP1/cep1::ura3 HIS4/his4 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)]
D63	[381G] MATa/MATa leu1/LEU1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1::LEU2 HIS4/his4 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)]
D64	[381G] MATa/MATa leu1/LEU1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1::LEU2 cep1::ura3/cep1::ura3 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)]
D68	[381G] MATa/MATa LEU1/leu1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)/CFIII(D8B.d.D30-18B.LEU2)]

TABLE 1 cont'd.

D69	[381G]MATa/MATa leu1/LEU1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/TYR1 TRP1/trp1 cep1::ura3/cep1::ura3 can1/CAN1 cyh2/CYH2 [CFIII(D8B.d.D30-18B)/CFIII(D8B.d.D30-18B.LEU2)]
D77-R1	MATa leu2 his3 arg4 petX [cep1::URA3]-10 ade1::HIS3/ade1::LEU2 (chr. I disome)
D92	MATa/MATa leu2/leu2 his3/his3 TRP1/trp1 ADE6/ade6 ARG4/arg4 PETX/petX CEP1/[cep1::URA3]-10 ADE1/ade1::HIS3/ade1::LEU2
D93	MATa/MATa leu2/leu2 his3/his3 TRP1/trp1 ADE6/ade6 ARG4/arg4 PETX/petX [cep1::URA3]-10 / [cep1::URA3]-10 ADE1/ade1::HIS3/ade1::LEU2
R31	MATa/MATa ura3/ura3 lys2/lys2 leu2/leu2 ade2-o/ade2-o HIS3/his3 trp1/trp1 CEP1/cep1::TRP1 CFVII(RAD2.d URA3 SUP11)
R31-1A	MATa ura3 lys2 leu2 ade2-o trp1 CEP1 CFVII(RAD2.d URA3 SUP11)
R31-3B	MATa ura3 lys2 leu2 ade2-o his3 trp1 cep1::TRP1 CFVII(RAD2.d URA3 SUP11)

^aAll except the last 6 strains listed are congenic to 381G (Hartwell 1980), and the genotypes given are in addition to the 381G markers.

sequences with *URA3*; their *cep1* phenotypes (see Chapter III) are indistinguishable. A *Ura*⁻ derivative of [*cep1::URA3*]-11 designated *cep1::ura3*, was obtained by selecting for 5-fluoroorotic acid resistance. The *cep1::TRP1* allele replaces internal *CEP1* coding sequences with *TRP1*; its phenotype (see Chapter III) is indistinguishable from *cep1::URA3*. R31 was constructed by *CEP1* gene transplacement in strain YPH98 using plasmid pRB101 (*cep1::TRP1*) as described (75). The *trp1::LEU2* allele was obtained by gene transplacement using plasmid pEL11 as described (59). Chromosome I trisomy was introduced via strain VG31-11C of (38). First, *CEP1* was disrupted to obtain strain D77-R1 (Table 1), then D77-R1 was mated with two different 381G-derived parents to obtain trisomic strains D92 and D93. D92 and D93 are thus congenic with each other, but not with the other 381G strains.

All media were as described (5) except for color indicator plates which were synthetic complete medium containing only 6 µg/ml adenine (1/3 normal concentration). Selection for *ura3* mutants was carried out with uracil dropout plates supplemented with 50 µg/ml of uracil and 1.0 mg/ml of 5-fluoroorotic acid (PCR, Inc., Gainesville, Florida) (9). Sporulation medium was 1% potassium acetate supplemented with adenine, histidine, lysine, tryptophan, tyrosine, leucine, uracil, and methionine at one half their normal concentrations. All strains were routinely grown at 30°. Sporulation was carried out at 22° and assays for temperature sensitive growth at 34°. Yeast transformations were performed by the lithium acetate procedure (51), as modified by Schiestl and Gietz (88).

Plasmids used for segregation analysis. Plasmid pDK243 (56) was obtained from D. Koshland, plasmid pJS2 (90) from P. Hieter, plasmid pEL11 (59)

from E. Louis, and plasmid pRIP1 (78) from R. Parker. Plasmid pDM8 was constructed by inserting (after Klenow fill-in) the 5.4-kbp *SalI-SmaI ade3-2p* fragment from pDK243 into the *SmaI* site of pJS2, inactivating the *SUP11* gene. Plasmid pDM2 was derived from pRIP1 by inserting a Klenow-blunted 2.2-kbp *LEU2* fragment into the *EcoRV* site in *URA3*.

Plasmids used for CP1 expression in yeast and *E. coli*. Figure 1 shows the two basic vectors used for expressing CP1. Plasmid pDR28ARS contains, in addition to *ARS1*, *CEN3*, and the selectable marker *LEU2*, an epitope-tagged *CEP1* gene (*CEP1 FLAG*). *CEP1 FLAG* differs from wild-type *CEP1* in that codons 1-9 were changed to encode MDYKDDDDK which specifies the FLAG™ epitope developed by Immunex Corporation (Seattle) (47). [The wild-type CP1 N-terminus is MNSLANNK.] The *HindIII* site encoded at codons 9/10 of wild-type *CEP1* is retained in the same reading frame in *CEP1 FLAG*.

The *E. coli* T7 expression vector pJH7 was derived from pET-3d (92) by destroying the *XbaI* and *HindIII* sites, ligating an *XbaI* nonsense linker (encoding translation stops in all three reading frames) to the *BamHI* site, and inserting *CEP1FLAG* in two steps. First, a 0.4-kbp segment of *CEP1 FLAG* was amplified by the polymerase chain reaction using an upstream primer to create an *NcoI* site at the initiator ATG, and subcloned into the pJH7 vector backbone. Then, all *CEP1* sequences except the FLAG codons were removed and replaced with the *HindIII*-*BglII* fragment of wild-type *CEP1*, using *XbaI* linkers at the *BglII* end.

Plasmids used for expression of mutant CP1 alleles. Unless stated otherwise, all of the *CEP1* mutant alleles were constructed from pDR28ARS, and then moved (as *HindIII*-*BglII* fragments) into pJH7 for expression in *E. coli*.

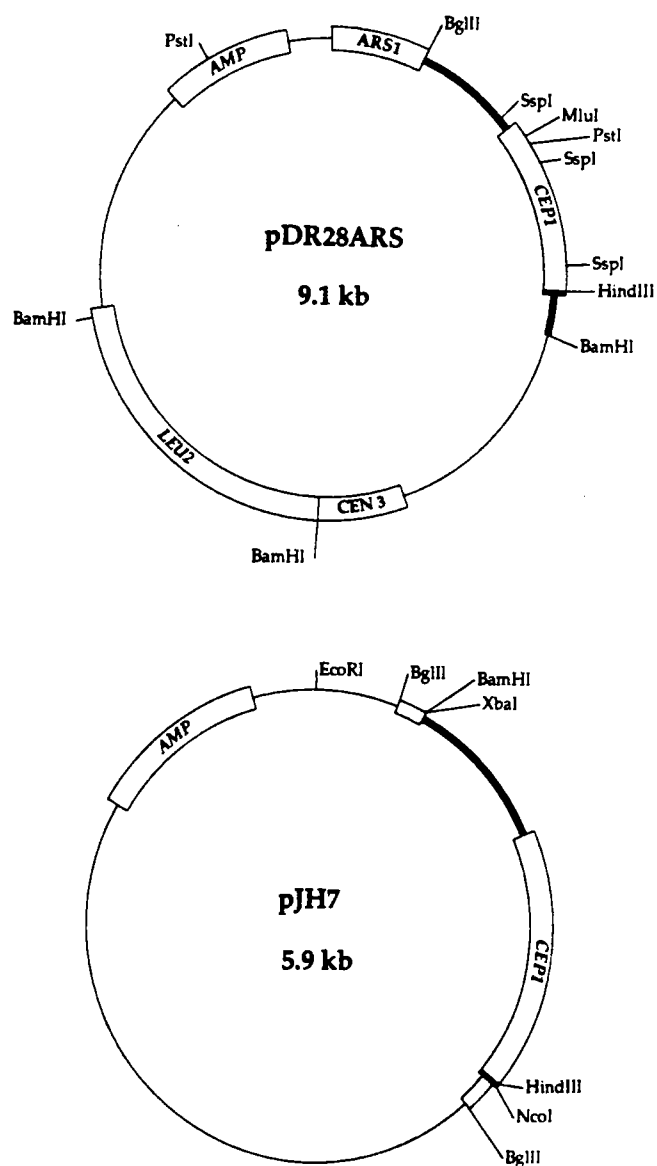


Figure 1. Plasmids used for expression of *CEP1* alleles in yeast (pDR28ARS) and *E. coli* (pJH7).

Yeast multicopy (YE_p) constructs were obtained by subcloning *CEP1* BamHI-BglII fragments into the BamHI site of YE_p351 (45); all YE_p plasmids have the *CEP1* segments in the same relative orientation. Table 2 cross-references the *CEP1* expression plasmids used for the mutational analysis described in Chapter V.

Linker insertion (LI-) alleles contain in-frame insertions at naturally occurring restriction endonuclease sites. The LI-301 allele was constructed by limited PstI digestion of pDR28ARS, removal of the 3' overhanging ends by using Klenow enzyme in the absence of dNTP's, and religation in the presence of 10-bp synthetic XhoI linkers (5'-CCCTCGAGGG-3'). Alleles LI-381PG and LI-381SR were constructed by digesting pDR28ARS with MluI, filling in the staggered ends with Klenow polymerase, and ligating in the presence of either 8 bp SmaI linkers (5'-GCCCCGGGC-3') or 8 bp XhoI linkers (5'-CCCTCGAGG-3'), respectively. The LI-259 allele was derived from the primary *CEP1* clone pDR4-2 (5) by partial SspI digestion and ligation in the presence of 12-bp SacII linkers (5'-TCCCCGCGGGGA-3'). One of the ligation products contained a single linker inserted at codon 259. The HindIII-BglII fragment of this plasmid was then used to replace that of pDR28ARS. DNA sequencing was performed on all of the mutant constructs to verify that the linkers had inserted as expected and the *CEP1* reading frame was preserved.

The R235H point mutant was obtained by hydroxylamine mutagenesis *in vitro* and plasmid shuffling (87). Mutagenized pDR28ARS DNA was used to transform an *ade2 ade3 cep1 leu2* strain (D1-1C) which contained a second plasmid (pDR33) carrying wild-type *ADE3* and *CEP1* genes. Transformants were selected on plates lacking leucine and methionine, and red,

TABLE 2
Plasmids used for mutational analysis

<i>cep1</i> Allele	Vector Backbone		
	pDR28ARS	pJH7	YEp351
Wild-type	pDR28ARS	pJH7	
NΔ33	pRB95-H23	—	—
NΔ86	pRB95-H10	—	—
NΔ137	pRB95-H24	—	—
NΔ162	pRB95-L2	—	—
NΔ208	pKO1-3	pJH9-4	pDM30
NΔ332	pRB95-L15	—	—
CΔ302	pJH11	pJH8-2	pDM25
LI-301	pDM16	pDM19-1	pDM26
LI-318PG	pRB103	pKO23	pDM23
LI-318SR	pRB104	pKO24	pDM24
LI-259	pDM13	pKO21	pDM31
R235H	pDM17	pDM20-1	pDM32
TFEB	pRB109	pRB108	pDM29

non-sectoring colonies were picked, *i.e.*, transformants requiring pDR33 (*CEP1*⁺) for a Met⁺ phenotype. Plasmids were recovered and sequenced. One, pHA4, contained two point mutations: a G to A transition at nucleotide position 959, and a G to T transversion at position 1175 (nucleotide coordinates of (5)). The mutations were separated by subcloning the HindIII-PstI and PstI-BglII fragments of pHA4 separately into pDR28ARS. The mutation at position 1175 had no observable phenotype, but the mutation at position 959 (R235H) resulted in loss of CP1 function.

The 5' deletions were made by exonuclease III digestion of HindIII-cut pDR4-2 using Erase-a-Base kit components (Promega) according to the manufacturers instructions. After ligation with HindIII linkers (GAAGCTTC), the DNA was cleaved with BglII and fragments were gel-purified and ligated to HindIII-BglII digested pDR28ARS. Deletion endpoints were determined by DNA sequencing. The 3' deletion allele, CA302, was obtained in a similar fashion except exonuclease digestion was initiated at the BglII site, 14 bp XbaI linkers (CTAGTCTAGACTAG) were added, and the resulting HindIII-XbaI fragment was ligated to HindIII-XbaI-cut pJH7 to produce pJH8. For expression in yeast, the CA302 segment was removed from pJH8 as a HindIII-BamHI fragment and ligated to HindIII-BglII cut pDR28ARS (pJH10). This plasmid transformed yeast poorly, presumably due to transcription through the adjacent ARS1 element. The problem was alleviated when 0.6 kbp of *CEP1* downstream sequences were reintroduced. This was accomplished by replacing the XbaI-EcoRI segment of pJH10 with the HindIII-EcoRI segment of the 5'-deletion mutant NA333 using an XbaI nonsense linker to adapt the HindIII end.

The resultant plasmid (pJH11) thus contains *CEP1* codons 333-351 immediately downstream of the *XbaI* nonsense linker which terminates translation.

TFEB expression plasmids all contain nucleotides 871 (*RsaI*) to 1523 (*SmaI*) of the TFEB cDNA isolated by Carr and Sharp (18). Using standard procedures, an 8-bp *HindIII* linker (GAAGCTTC) was added at the 5'-end and a 14-bp *XbaI* linker (CTAGTCTAGACTAG) added at the 3'-end. This segment was then inserted into pJH7 for *E. coli* expression and into the pJH11 vector backbone for expression in yeast.

Generation of chromosome fragments. Chromosome fragment CFIII(D8B.d.D30-18B) was generated by transforming strain D30-18B with *NotI*-cut pDM8 and selecting for uracil prototrophy (37). Several of the transformants had the expected phenotype. They formed pink colonies with rare white sectors, the result of mitotic loss of the chromosome fragment. The *Ura* and color phenotypes cosegregated. The expected structure of CFIII(D8B.d.D30-18B) is a long arm consisting of the left arm of chromosome III distal to the D8B sequence, the centromere region from chromosome VI, and a short arm consisting of vector sequences, *URA3*, and *ade3-2p* (90). Its size is about 150 kbp and for convenience we have designated it CF(*URA3*). A *LEU2* derivative of CF(*URA3*) was obtained by marker change generating CFIII(D8B.d.D30-18B.*LEU2*). The marker change was accomplished by one-step gene disruption using the *ura3::LEU2* disruption allele of plasmid pDM2. Strain D64-61A was transformed with *NsiI*-*NdeI*-cut pDM2 DNA selecting for leucine prototrophy. Among the transformants were several that were phenotypically *Leu*⁺ and *Ura*⁻ and formed pink colonies with rare white sectors; the *Leu* and color phenotypes cosegregated. For convenience,

CFIII(D8B.d.D30-18B.*LEU2*) is referred to as CF(*LEU2*). The structures of CFIII(*URA3*) and CF(*LEU2*) were verified by pulsed field gel electrophoresis and Southern blotting (data not shown).

Measurement of mitotic loss rates. Mitotic plasmid loss and nondisjunction rates were measured by the half-sectored colony assay described by Koshland and Hieter (55).

Mitotic CF loss and nondisjunction rates were determined by fluctuation analysis as described (43) except only two 150-mm indicator plates spread with approximately 1500 cells were used for each resuspended colony. The total CF loss rate was calculated based on the number of white colonies arising in the population. The CF nondisjunction rate (2:0 segregation) was determined based on the appearance of red colonies (90). The rate of simple loss (1:0 segregation) is the total loss rate minus the rate of nondisjunction. For assays where pDR28ARS and YEp351 derived plasmids were used to express CP1 in trans, leucine was omitted from all media to maintain selection for the plasmid. In these cases, the tester strain, R31-3B (*ade2-o ADE3 leu2 ura3*), carried a nonessential chromosome fragment CFVII(*RAD2.d.URA3 SUP11*), referred to as CFVII, whose ploidy could be determined by colony color phenotype based on the suppression of the *ade2-o* allele by *SUP11* (55).

Analysis of meiotic segregation of plasmids and CFs. Diploids containing plasmids or chromosome III derived CFs were grown selectively, otherwise they were grown in YEPD. Cells from fresh overnight cultures were washed with 1% potassium acetate, resuspended in sporulation medium at a density of 1×10^7 cells/ml, and incubated at 22° for 4-5 days. Sporulation efficiency of both wild-type and *cep1* strains was 15-45%. Tetrads were dissected

onto YEPD agar slabs and incubated for 4-5 days at which time the colony color phenotype could be scored. Cells from each colony were then transferred to a YEPD master plate. After 24 hours of growth the patch cultures were transferred to nutritionally defined media and drug-containing media to score the various phenotypes. Mating of the segregants was determined by using replicaplate transfer of the master plates to lawns of mating type tester strains (*MATa hom3* or *MAT α hom3*); after overnight growth diploid formation was detected by replicaplate transfer and scoring complementation of the *hom3* marker.

The *ade2/ade3-2p* system described by Koshland *et al.* (56) was used to monitor the copy number of plasmids and CFs. Cells lacking plasmid or CF form white colonies. Cells containing one copy of the plasmid/CF form predominantly pink colonies with occasional white and red sectors resulting from mitotic loss/nondisjunction of the plasmid/CF. Cells with two or more copies of the plasmid/CF form predominantly red colonies with occasional pink and white sectors. Color phenotype was most easily scored on defined media containing one third the normal amount of adenine, although scoring on standard YEPD (no added adenine) was also possible. Also, red colonies tend to grow more slowly than pink ones due to the increased accumulation of the red pigment which is somewhat toxic. In all cases where red spore colonies were identified, the presence of more than one plasmid/CF was verified genetically by crossing cells of the red colony to testers containing no plasmid/CF. The resulting diploids were induced to sporulate, asci were dissected, and haploid segregants were scored for segregation of the plasmid/CF.

When the original parent contains two copies of the plasmid/CF, it segregates predominantly 4:0 in the test cross.

Sister spores were identified by scoring *trp1* which is located 0.4 cM from *cen4* (70). In experiments involving CFs, the *cen7*-linked marker *leu1* was also present. Segregation of the CF was scored first against *trp1* and then verified against *leu1*. To be counted as a CF nonsister segregation event, the CF must have displayed second division segregation with respect to both centromere-linked markers. Tetrads in which sister segregation of the CF was ambiguous were eliminated from the analysis. Since the second division segregation frequencies of *trp1* and *leu1* are 0.94% and 4.9%, respectively (70), the expected frequency of simultaneous second division segregation of both markers is less than 0.05%. The observed frequency of tetratypes for *trp1* and *leu1* in our wild-type strains was 5.0% (ditype:tetratype, 151:8), very close to the value which would be predicted for these two markers based on their reported second division segregation frequencies. In the *cep1* mutants, the tetratype frequency for *trp1* and *leu1* is increased to 9.6% (ditype:tetratype, 189:20). This difference is not statistically significant by Chi-square test ($P > 0.05$), and it is not nearly sufficient to account for the increased nonsister segregation of CFs observed in *cep1* strains.

Screen for chromosome disomy among meiotic segregants. The procedures of Louis and Haber (59) were used to screen for disomy of chromosomes *III*, *IV*, *V*, and *VII*. Haploid spores disomic for one of the marked chromosomes are recognized by the following phenotypes: Chromosome *III* disomes (*MATa/MAT α*) are non-mating and non-sporulating. Chromosome *IV* disomes (*TRP1/trp1::LEU2*) are both *Trp*⁺ and *Leu*⁺. Chromosome *V* disomes

(*CAN1/can1*) form colonies which when replicated to canavanine plates are drug-sensitive but which give rise to canavanine-resistant papillae resulting from mitotic loss of the *CAN1* homolog or from *can1/can1* mitotic recombinants. Likewise, chromosome VII disomes (*CYH2/cyh2*) give rise to papillating colonies on cycloheximide plates. The latter two phenotypes are easily detected in a *cep1* background where mitotic chromosome loss is increased. In all cases, the screens detect only disomes carrying heterozygous markers; therefore, some missegregation events are missed due to recombination between the marker and its centromere which produce a homozygous disome. For details on these screening procedures, see Louis and Haber (59).

Chromosome I disomy in segregants of strains D92 and D93 was identified by scoring the *cen1*-linked markers *ADE1*, *ade1::HIS3*, and *ade1::LEU2*. Haploid spores contain only one of the three markers, while disomes contain two. [For the D92 segregants, *ADE1* was scored by complementation using *ade1* testers.] 2:2 segregation of all three markers was observed in 98% of the tetrads; concomitant 3:1/1:3 segregation for two of the markers was observed in the remainder. Since four CEN-linked markers were segregating (three *ADE1* alleles in addition to *trp1*), sister spores could be determined unambiguously. Recombination in the *CEN1-ADE1* interval was observed at a frequency of about 10% (21/201), and in the *CEN4-TRP1* interval at 0.5% (1/201). No significant difference was observed in recombination frequency between the wild-type and *cep1* strain.

Electrophoretic karyotyping of meiotic segregants. DNA was prepared for CHEF gel analysis as described by Mortimer (69). 2×10^8 cells from overnight cultures grown in YEPD were pelleted, washed in 0.125M EDTA

(pH 8), and suspended in 0.5 ml of SCE [1M sorbitol, 0.1M Na-Citrate, 10mM EDTA(pH 7.5)] containing 1mg/ml of zymolyase (Seikagaku Kogyo Co., LTD.). Cells were incubated at 30° for 30 minutes without shaking, pelleted and suspended 0.2 ml of 1M sorbitol, 0.1M EDTA (pH 8). To each sample, 0.2 ml of 1.5% molten (45°) LMP agarose (in 0.125M EDTA) was added by gently pumping up and down using a pipetteman fitted with a sawed-off tip, and 50 µl aliquots were transferred onto Parafilm. Solidified "plugs" were transferred to 3 ml capped polypropylene tubes to which 1.5 ml of 1% sarkosyl, 0.5M EDTA (pH9.5), containing 1 mg/ml proteinase K was added. Tubes were rocked gently overnight and plugs were then rinsed three times in 10mM Tris, 50mM EDTA (TE50) and then dialyzed 4-5 times for 45-60 minutes (~ 4 hours total) in 2 ml of TE50. Plugs were stored at 4° in TE50 until use.

For CHEF analysis, plugs were melted at 65° and loaded into wells of a 1% agarose gel (in tank buffer) which was run at 200V (~100mA) in a Hex-A-Field CHEF gel apparatus (GIBCO BRL) for 24 hours at 15° in 1/2X TBE buffer. The switch time was 60 s for the first 14 hours and 90 s for the remaining 10 hours. Gels were stained with ethidium bromide and photographed. The amounts of DNA in the chromosome bands were quantitated by densitometric scanning of photographic negatives of the gels. The relative DNA quantities were determined by first normalizing the values obtained for the different chromosomes within a given lane to a reference chromosome within that lane, and then correcting these values for the molecular weights of the chromosomes.

Assays for methionine-independent growth. A semi-quantitative assay was devised to score methionine-independent growth. Several independent

transformant colonies of each strain were grown as patches of cells at 30° on plates lacking leucine, then replica plated in triplicate onto -Leu dropout and onto -Leu -Met double dropout plates. The plates were incubated at 22°, 30°, and 34° and scored after 24, 48, and 72 hours. On each day, the patches were assigned a score of 2 (confluent growth), 1 (less than confluent growth) or 0 (no detectable growth), and the individual scores added after 3 days. Thus, the maximum score is 6 and the minimum 0. Wild-type cells routinely gave a score of 6 and *cep1* mutants a score of 0.

Preparation of yeast and *E. coli* extracts. Sources for the yeast extracts were strains (R31-3B) carrying CP1 expression plasmids which had been grown at 30° in 100 ml of selective (-Leu) medium to mid log phase. All subsequent manipulations were carried out on ice or at 4°. The cells were washed once with water, resuspended in two pellet volumes of 200mM Tris (pH8.1)-10mM MgCl₂-10mM β-mercaptoethanol-10% glycerol-1mM PMSF, transferred to 2-ml screw cap microfuge tubes containing 1 ml of 0.5-mm glass beads, and broken by two-20 s bursts in a Biospec Products Mini-Bead Beater. Tubes were cooled for 20 s in an ice-ethanol bath between agitations. Lysates were transferred to 1.6-ml tubes and spun in a microcentrifuge for 10 min to remove cellular debris. Supernatants were frozen in aliquots on dry ice and thawed only once before use. Protein concentrations were determined by the method of Bradford (10) using bovine serum albumin as standard.

Soluble *E. coli* extracts were prepared essentially as described by Studier et. al. (92). The host strain for T7-induced expression was BL21(DE3) carrying the plasmid pLysS. BL21(DE3) transformants carrying pJH7-derived CP1 expression plasmids were grown overnight at 37° in LB broth containing ampi-

cillin and chloramphenicol. The overnight cultures were diluted 1:100 into fresh medium and grown at 37° to an OD₆₀₀ of 0.3-0.6 at which time isopropylthiogalactoside (IPTG) was added to a final concentration of 0.4mM.

Incubation was continued for an additional 90 min. Cells were pelleted, re-suspended in extract buffer [200mM Tris (pH8.1), 10mM MgCl₂, 10mM β-mercaptoethanol, 10% glycerol, 0.5mM PMSF, 0.5mM DTT, 0.2% NP40] to a final concentration of 4 OD₆₀₀ units per ml, and chilled on ice. Cell breakage was accomplished by sonication using a Branson J-17A sonicator at full power for two 30 s bursts with a 30 s rest on ice between disruptions. After pelleting cellular debris by centrifugation for 10 min at 4° in a microcentrifuge, the supernatants were transferred to clean tubes and frozen on dry ice.

Quantitation of CP1 in *E. coli* extracts. Equal volumes of CP1-expressing *E. coli* extracts were run on 12% SDS polyacrylamide (Laemmli) gels and the gels stained with Coomassie blue. The CP1 bands were clearly detectable above the background of *E. coli* proteins. After destaining, the gels were sandwiched between two sheets of 3M transparency film and scanned using a Hoeffer Scientific GS-300 Densitometer. Peak integration was accomplished using GS-365 PS Electrophoresis Data System software (Hoeffer). Gel lanes of extracts of *E. coli* not carrying CP1 expression plasmids were scanned to determine background staining in the regions of the gels containing CP1 products. This background accounted for less than 10% of the integrated CP1 signal in all cases except for LI-318SR which was poorly expressed. Background-subtracted CP1 integration values were corrected for differing polypeptide molecular weight and then normalized to the CP1 signal obtained from a single extract of the NA208 mutant, an aliquot of which was included on all gels

for standardization. Four separate scans of samples from at least two independent extracts were done for each CP1 protein. Results from duplicate scans differed by less than 10%.

The absolute concentration of CP1 in the extracts was estimated by running the "standard" NΔ208 extract on a gel along with known amounts of homogeneously pure human serum albumin (a gift of R. Feldhoff, University of Louisville School of Medicine). After densitometric analysis as described above, the concentration of NΔ208 polypeptide was determined from a standard curve. The "standard" extract contained 0.47 mg/ml of NΔ208 polypeptide (29 μM).

Gel shift DNA binding assays. Probe for DNA binding assays was prepared from plasmid pDR1-7 which contains a single copy of the CDEI oligonucleotide 5'-GATCCAAATAAGTCACATGATGATA(GATC)-3' cloned into the BamHI site of pUC18. After EcoRI/HindIII digestion and Klenow fill-in with [α - 32 P]dATP, the 72-bp probe was separated by polyacrylamide gel electrophoresis and isolated by electro-elution. Probe DNA concentration was determined using a Hoeffer Scientific model TKO 100 DNA fluorimeter. Alternatively or in addition, samples of probe DNA were run on a 6% polyacrylamide gel along with known amounts of EcoRI-HindIII digested pDR1-7 plasmid DNA and DNA concentration determined after ethidium bromide staining (4).

Gel shift DNA binding assays were performed as described (3). Binding reactions contained (in 15 μl) 20mM Hepes, 150mM KCl, 5mM MgCl₂, 2.5% glycerol, 0.25 mg/ml bovine serum albumin, 0.05% Nonidet P-40, 20,000 cpm of 32 P-labeled probe DNA (200-500 pg), unlabeled competitor DNA, and either

yeast or *E. coli* extract. Assays using *E. coli* extract contained 1 μ l of a 1:100-diluted extract (30-60 ng total protein) and 2 μ g of poly d(I-C) as unlabeled competitor. Assays using yeast extract contained 20 μ g total yeast protein (2-5 μ l extract) and either 2.5 μ g pDR1-12 plasmid DNA (specific competitor) or 2.5 μ g pDR2-14 plasmid DNA (nonspecific competitor). After incubation for 15 min at room temperature, binding reactions were electrophoresed on 4% polyacrylamide gels which were subsequently dried and exposed to X-ray film. The specific competitor DNA was plasmid pDR1-12 which contains a pentamer of the CDEI oligo described above in the BamHI site of pUC18. Nonspecific competitor was plasmid pDR2-14 which is identical except that it contains a pentamer of an unrelated oligonucleotide (5'-GATCCGTTTTCCGAAGATGT-3').

Determination of relative apparent binding constants. Dried binding assay gels were analyzed on a Betagen Betascope 603 blot analyzer to quantify the amount of bound and free probe in each binding reaction. Relative apparent CP1 binding constants (K_{rel}) were calculated using the following equation:

$$K_{rel} = \frac{K_{test}}{K_{ref}} = \frac{R_{test}}{R_{ref}} \cdot \frac{[CP1]_{ref}}{[CP1]_{test}}$$

K_{test} and K_{ref} are the apparent binding constants of, respectively, the test and reference proteins; R_{test} and R_{ref} are the ratios of bound to free probe for the respective binding reactions; $[CP1]_{test}$ and $[CP1]_{ref}$ are the total concentration of test and reference proteins in the reactions, respectively. The standard NA208 extract was used as the "reference" in all cases. The above equa-

tion assumes that the concentration of unbound CP1 protein in each binding reaction is approximately equal to the input concentration of CP1, *i.e.*, conditions of protein excess. This condition was met in our reactions where the total CP1 concentration was 10-20nM and the concentration of bound probe was 0.01-0.15nM.

Figure 2 shows the results of a typical DNA binding assay. The fastest migrating bands are free probe and the slowest migrating bands are CP1-DNA complex. The band of intermediate mobility is specific and probably due to a CP1 degradation product. The intermediate-mobility bands were ignored when estimating K_{rel} ; however, since these bands accounted for at most 20% of the total bound DNA, their omission made no significant difference in the calculations. Each of the entries in Table 10 are the mean determination for two or three independent extracts of each protein. The average deviation from the mean was 20%.

Immunodetection of FLAGged CP1 proteins. Crude yeast proteins (up to 30 μ g of total protein) were resolved on 12% polyacrylamide SDS (Laemmli) minigels (0.75mm). Proteins were transferred to Immobilon PVDF membranes (Millipore) in 1X CAPS buffer containing 10% methanol (61). Detection of FLAGged proteins was carried out using the Amersham ECL detection system following the manufacturers guidelines. The primary antibody used was monoclonal anti-FLAG-M5 mouse IgG1 diluted to 10 μ g/ml in 0.1%TTBS,pH7.5 (0.1%Tween-20, 20mM Tris-HCl, 0.5M NaCl). The secondary antibody was peroxidase-conjugated goat anti-mouse Ig

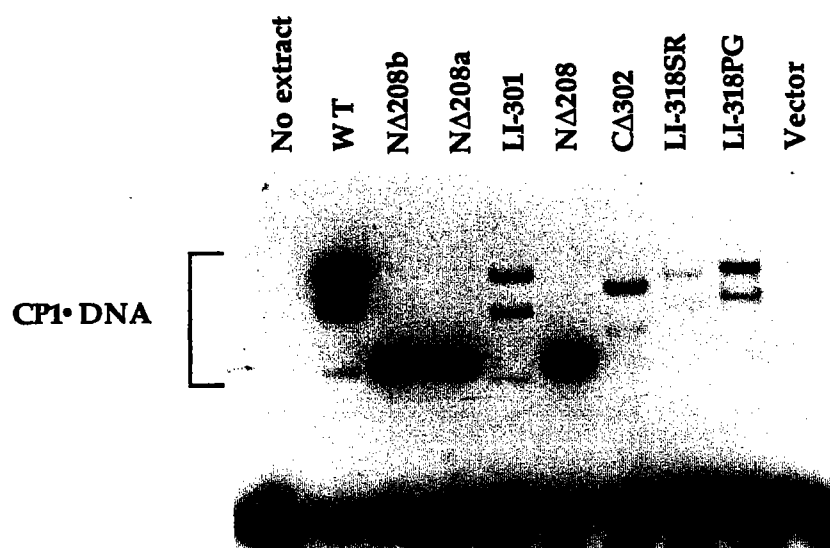


Figure 2. Representative DNA binding assay of *E. coli*-expressed CP1 proteins. Assays were carried out using 1 μ l of *E. coli* extract as described in Materials and Methods. The CA302 and LI-301 extracts were diluted 1/25 and the others 1/100. The three NΔ208 reactions utilized independent extracts.

(Boehringer Mannheim Biochemicals) diluted 1:3,000 in 0.1%TTBS containing 2.5% dry milk. After reaction with antibody, membranes were washed three times in 0.1%TTBS-0.1% dry milk. For maximum sensitivity, a single overnight exposure of the blot was made.

CHAPTER III

CLONING, GENE DISRUPTION, AND MAPPING OF *CEP1*

(Baker, R. B., and D. C. Masison. 1990. Isolation of the gene encoding the *Saccharomyces cerevisiae* centromere-binding protein CP1. *Mol. Cell. Biol.* 10: 2458-2467.)

Cloning of *CEP1*. The gene encoding CP1 was isolated by using DNA hybridization to screen a λ gt11 yeast genomic library. The oligonucleotide probes were designed based on peptide sequence information that was derived from purified CP1 (3, 57). Details of the isolation and cloning of *CEP1* are contained in Chapter II. The DNA sequence of the *CEP1* gene along with the deduced amino acid sequence is shown in Figure 1 of the appendix. *CEP1* encodes a 351 amino acid polypeptide of molecular weight 39,400 which is highly acidic, having a predicted net charge of -20 at pH 7. The gene sequence reveals a conspicuous absence of cysteine residues which is consistent with the observation that a low content of cysteine residues (<0.3%) is a general feature of proteins implicated in the regulation of sulfur amino acid metabolism (96).

Verification of *cep1* null mutant phenotypes. Strains harboring null mutations of *CEP1* display three major phenotypes: a 35% increase in cell doubling time, a ninefold increase in mitotic chromosome loss rate, and methionine auxotrophy (5). Our published results describing these phenotypes were obtained using strains carrying a *cep1* disruption allele ([*cep1::URA3*]-11, Figure 3) in which several hundred base pairs of 3' flanking DNA were deleted. Since yeast genes are often in close proximity, a question arose as to whether any of the phenotypes observed for strains carrying this allele were caused by mutation of a neighboring gene.

Two approaches were used to verify that the phenotypes we observed for the strains carrying the [*cep1::URA3*]-11 allele were not due to the deletion of the 3' flanking DNA. First, a disruption allele which removes *CEP1* coding region and only 90 bp of 3' flanking DNA ([*cep1::URA3*]-10, Figure 3) was used

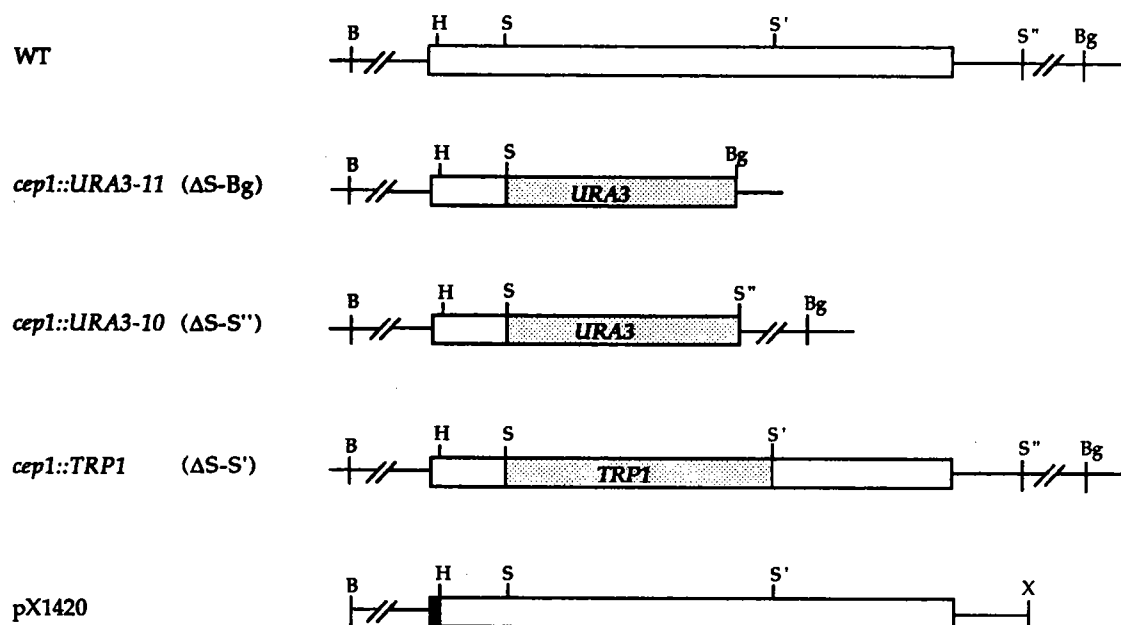


Figure 3. Wild type and disruption alleles of *CEP1* (Not to scale). The coding region of *CEP1* is diagrammed as an open box with the relevant restriction endonuclease sites indicated (B, BamHI; Bg, BglII; H, HindIII; S, S', S'', SspI). The BamHI, HindIII, and SspI sites correspond to those in the *CEP1* DNA sequence shown in Figure 1 of the Appendix, and the BglII site is located about 600 bp from the end of the coding region. The three disruption alleles contain replacements of the *CEP1* sequences deleted between the indicated restriction sites with the selectable marker genes (shaded) shown. The insert fragment of plasmid pX1420 (BamHI-XbaI) was derived from pDR28ARS (Figure 1) and contains the *CEP1FLAG* gene with 255 bp of 5', and 110 bp of 3' flanking DNA.

to create a new null mutation in the diploid strain R10 [R10 is isogenic with R11 (5) except for the *cep1* disruption allele]. Haploid derivatives of R10 carrying this allele were shown to have phenotypes indistinguishable from haploid strains carrying the *cep1::URA3-11* allele (not shown). Second, a plasmid-born *CEP1* allele containing only 110 bases of 3' flanking DNA (pX1420, Figure 3) was shown to be able to complement all of the phenotypes of the *cep1::URA3-11* null mutant strains (not shown).

The *cep1::TRP1* allele (Figure 3) provided further evidence that the mutant phenotypes were due to the lack of CP1. Tetrad analysis of strain R31 (*cep1::TRP1* /+) is shown in Figure 4. Reduced growth rate, indicated diagrammatically by the smaller size of the dissected spore colonies (top panel of Figure 4) segregated with the *TRP1* disruption of *CEP1*. In addition, all segregants inheriting the *cep1::TRP1* allele were unable to grow in the absence of methionine. Compared with the wild type colonies, the *cep1::TRP1* colonies also display an increased frequency of mitotic loss of the chromosome fragment CFVII which is observed visually as increased sectoring of the colonies from white to red (see Chapter II). Based on these results, we conclude that the three phenotypes we describe for *cep1* null mutant strains, regardless of the disruption allele used to create the null mutation, are attributed solely to the loss of functional CP1.

Three point mapping of the *CEP1* genomic locus. Tetrad analysis indicated that *CEP1* was located very close to *SUP4* on chromosome X (5). In order to more precisely localize *CEP1*, I constructed strain D21. D21 carries the

ade2-o ura3 trp1Δ CEP1 CFVII(SUP11-URA3)
ade2-o ura3 trp1Δ cep1::TRP1

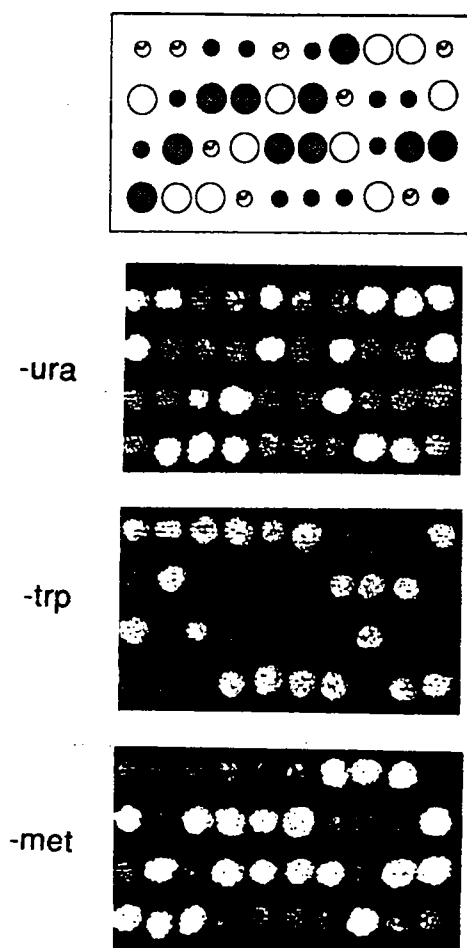


Figure 4. Phenotypes of *cep1* null mutants. The relevant genotype of diploid strain R31 is shown at the top of the figure. A master plate (YEPD) of haploid segregants was replica-plated onto Trp⁻, Met⁻, and Ura⁻ dropout plates. Photographs were taken after 24 hours incubation at 30°. The top panel diagrammatically represents a slab of dissected spore colonies from which cells were transferred in a similar pattern to the master plate. Each column represents the colonies arising from the four spores of a different tetrad. The smaller (slower growing) colonies are all *cep1::TRP1*. Segregants inheriting CFVII (Ura⁺) are white while the others are red. The increased mitotic loss of the CF for the *cep1* strains, resulting in red sectoring within the colony, is indicated by lines within the representative colonies.

cep1::URA3-11 and *SUP4* alleles, in which the *STE18* gene, which resides 32 centiMorgans distal to *SUP4* (71), was disrupted by the selectable marker *LEU2*. Tetrad analysis of strain D21, shown in Table 3 and included in the eleventh edition of the yeast genome map (68), localized *CEP1* to a position 4.6 centiMorgans distal to *SUP4* on the right arm of chromosome X.

Mitotic stability of a CEN plasmid and a CF in *cep1* strains. The increased mitotic chromosome loss seen for *cep1* strains could represent either the loss of a chromosome from the nucleus, or the failure of replicated chromosomes to segregate from each other. In order to examine this phenotype in more detail, the rate and type of missegregation of CEN plasmid pDK243 and chromosome fragment CF(URA3) were determined for wild type and *cep1* strains. The plasmid carries the selectable marker *LEU2*, the CF carries the selectable marker *URA3*, and both elements carry the *ade3-2p* allele to assay copy number. The results are shown in Table 4. The plasmid is significantly less stable than the chromosome fragment, but relative to wild-type cells the *cep1* mutants displayed increased mitotic loss rates of both, 3.2- and 25-fold, respectively. The ability to monitor copy number allowed distinction between simple loss (1:0 segregation) and nondisjunction (2:0 segregation) events. In *cep1* mutants, both 1:0 and 2:0 components of mitotic loss were increased, but in all cases, even in wild-type cells, the majority of loss events resulted from 2:0 rather than 1:0 segregation.

TABLE 3
Tetrad analysis

Diploid	Interval	No. of asci			Map Distance ^a (centimorgans)
		PD	NPD	T	
D21	<i>cep1::URA3-ste18::LEU2</i>	31	0	66	34
D21	<i>cep1::URA3-SUP4</i>	88	0	9	4.6
D21	<i>ste18::LEU2-SUP4</i>	27	0	70	36

^aMap distance = $\{[T+6(NPD)]/[2(PD+NPD+T)]\} \times 100$

TABLE 4
Mitotic loss rates of pDK243 and CF(URA3)

Strain	Element	Loss Rate ^a	Segregation	
			1:0	2:0
D24 (<i>CEP1/CEP1</i>)	pDK243	2.0×10^{-2}	$< 5.9 \times 10^{-4}$	2.0×10^{-2}
D25 (<i>cep1/cep1</i>)	pDK243	6.5×10^{-2}	1.6×10^{-2}	4.9×10^{-2}
D47 (<i>CEP1/CEP1</i>)	CF(URA3)	3.0×10^{-4}	0.6×10^{-4}	2.4×10^{-4}
D45 (<i>cep1/cep1</i>)	CF(URA3)	7.5×10^{-3}	0.6×10^{-3}	6.9×10^{-3}

^aEvents/cell/division

CHAPTER IV

MEIOSIS IN *SACCHAROMYCES CEREVISIAE* MUTANTS LACKING THE CENTROMERE-BINDING PROTEIN CP1

(Masison, D. C. and R. E. Baker. 1992. Meiosis in *Saccharomyces cerevisiae* mutants lacking the centromere-binding protein CP1. *Genetics* **131**: 43-53.)

Despite the lack of CP1, *cep1* strains maintain a relatively high fidelity of mitotic chromosome transmission. However, preliminary observations indicated that the overall viability of spores derived from homozygous *cep1* diploids was significantly reduced, suggesting that meiosis in yeast may be more sensitive to a deficiency of CP1. In support of this idea, both Cumberledge and Carbon (23) and Gaudet and Fitzgerald-Hayes (36) observed that plasmids containing CDEI-deleted centromeres displayed significantly increased rates of meiotic missegregation, more than would have been expected given the mitotic effects of the same mutations. The segregational defect observed was precocious sister segregation at the first meiotic division, suggesting that CDEI, and by inference CP1, were somehow involved in the maintenance sister chromatid cohesion during meiosis I (23, 36). The *cep1* null strains provided the opportunity to study the meiotic role of CP1 directly.

Meiotic segregation of pDK243 and CFs. Normally, an unpaired plasmid or CF segregates 2⁺:2⁻ at meiosis (Figure 5A). The replicated sister plasmids or CFs remain together through meiosis I and then segregate to sister spores at meiosis II. Precocious sister segregation at meiosis I results in segregation to nonsister spores, while meiosis II nondisjunction results in 1⁺:3⁻ segregation with one spore receiving two copies of the plasmid/CF. Plasmid/CF loss at either meiosis I or meiosis II also results in 1⁺:3⁻ segregation, but in this case the plasmid/CF-containing spore retains only a single copy of the plasmid/CF.

Table 5 shows the meiotic segregation analysis for plasmid pDK243. Assuming that tetrads segregating plasmid 4:0 and 3:1, 2:2 and 1:3, and 0:4 (plasmid⁺:plasmid⁻) arose from cells containing, respectively, two or more,

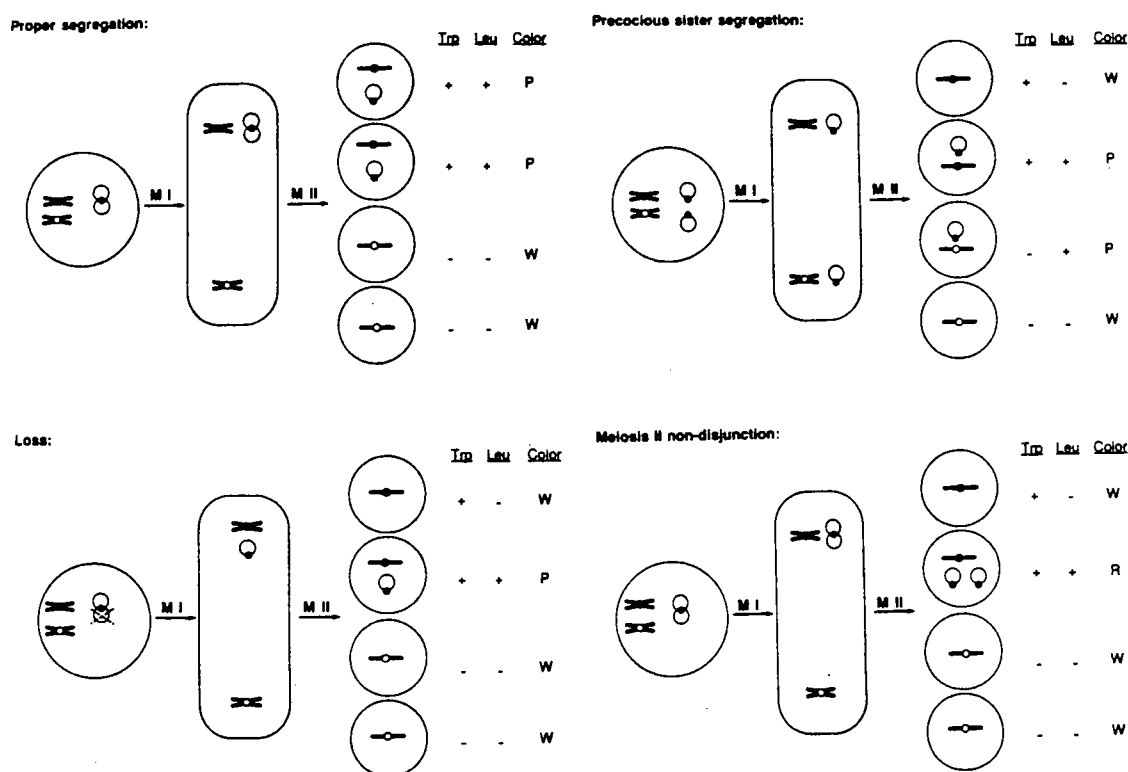


Figure 5A. Meiotic segregation of CEN plasmids. The chromosome represents chromosome IV which is used to mark sisters (*trp1/TRP1*). The circles represent plasmids (maintained at single copy) which carry the color marker *ade3-2p* and the selectable marker *LEU2*. Proper segregation results in 2⁺:2⁻ segregation of the plasmids to sister spores. Precocious sister segregation results in 2⁺:2⁻ segregation of the plasmids to nonsister spores. Meiosis II nondisjunction results in 1⁺:3⁻ plasmid segregation where the plasmid carrying spore is red, while simple loss results in 1⁺:3⁻ plasmid segregation where the plasmid carrying spore is pink. Segregation patterns of other unpaired elements (CFs, chromosome I of strains D29 and D93) are identical to those of the plasmid.

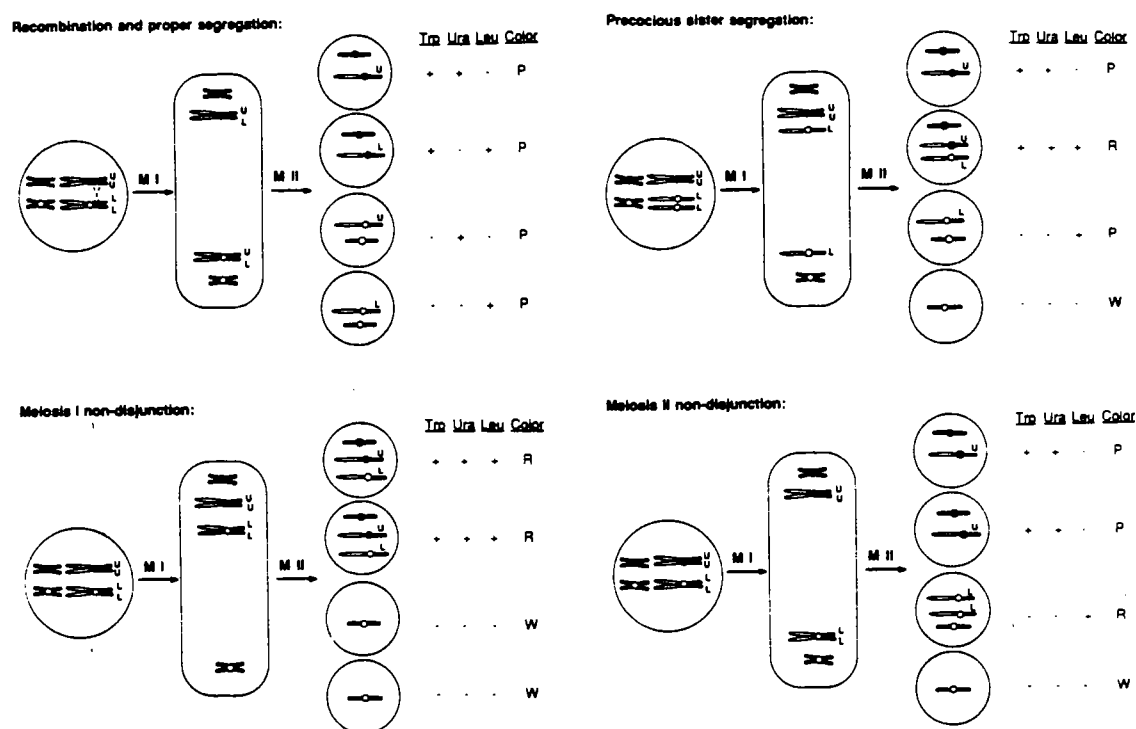


Figure 5B. Meiotic segregation of paired CF's. The smaller chromosome represents chromosome IV which is used to mark sisters (*trp1/TRP1*). The larger chromosome represents a nonessential CF which carries the color marker *ade3-2p* and either *URA3* (U) or *LEU2* (L) on its short arm. Recombination between the centromere and the short arm marker results in 4⁺:0⁻ segregation of the CF with both *URA3* and *LEU2* markers segregating to nonsister spores. (In the absence of recombination both markers segregate to sister spores). Precocious sister segregation results in 3⁺:1⁻ segregation with one CF disome (red) and one nullosome (white) occurring as nonsister spores. Meiosis II nondisjunction also results in 3⁺:1⁻ CF segregation, but in this case the disome and nullosome are sisters. Meiosis I nondisjunction results in 2⁺:2⁻ segregation with the production of two sister spore disomes (red) and two sister spore nullosomes (white). The Ura and Leu phenotypes indicated for the spores resulting from aberrant events assume no recombination along the short arm.

TABLE 5
Meiotic segregation of unpaired chromosomal elements

Strain	Element	Distribution in tetrads ^a						Segregation ^b		
		4:0	3:1	2:2	1:3	0:4	Total	S	NS	%NS
D24 (<i>CEP1/CEP1</i>)	pDK243	35	5	87	0	10	137	86	1	1.2
D25 (<i>cep1/cep1</i>)	pDK243	37	14	75	7	30	163	65	9	12.2
D30 (<i>CEP1/cep1</i>)	pDK243	0	0	31	0	0	31	30	1	3.2
D63 (<i>CEP1/CEP1</i>)	CF(<i>URA3</i>)	0	0	110	0	0	110	109	1	0.9
D64 (<i>cep1/cep1</i>)	CF(<i>URA3</i>)	5	7	104	12	13	141	80	21	20.8
D62 (<i>CEP1/cep1</i>)	CF(<i>URA3</i>)	0	0	23	0	0	23	23	0	0.0
D92 (<i>CEP1/cep1</i>)	Chrom. I	0	0	109	0	0	109	109	0	0.0
D93 (<i>cep1/cep1</i>)	Chrom. I	0	0	92	0	0	92	85	7	7.6

^aTetrads with 4 viable spores.

^bSister spores were identified by segregation of *trp1* (D24, D25, D30), *trp1* and *leu1* (D62, D63, D64), or *trp1* and *ade1*, (D92, D93). One D25 and three D64 tetrads were eliminated from consideration because sister spores could not be determined unambiguously. S = Sister, NS = Non-Sister.

one, or no copies of the plasmid before entering meiosis, the relative distribution of tetrads within each class closely reflected the distribution of plasmid in the premeiotic population (not shown). Only tetrads of the 2:2 and 1:3 classes were informative with respect to precocious sister segregation, meiosis II nondisjunction, or loss. Of 137 wild-type tetrads (strain D24, *CEP1/CEP1*), no cases of 1:3 segregation were observed and in tetrads segregating the plasmid 2:2, normal segregation of the plasmid was observed in 86 out of 87 cases. It is probable that the one case of nonsister segregation (0.9%) observed was not the result of plasmid missegregation but was due to a second division segregation of the centromere-linked *trp1* marker used to identify sister spores. [The reported second division segregation frequency of *trp1* is 0.9% (70).] For the *cep1* diploid (strain D25, *cep1/cep1*), there was a significant decrease in the number of tetrads segregating plasmid 2:2 and increases in the 3:1, 1:3, and 0:4 classes. The most significant difference was in the frequency of nonsister plasmid segregation. Nine of 74 tetrads (12%) segregated the plasmid to nonsisters. In addition, 6 loss events and one meiosis II nondisjunction were observed among the 163 meioses analyzed.

The segregation pattern of CF(*URA3*) is also shown in Table 5. The fidelity of CF(*URA3*) segregation in the wild-type strain is extremely high. One hundred percent of tetrads segregated the fragment 2:2 and only one case (0.9%) of segregation to nonsister spores was observed. This one case of nonsister CF(*URA3*) segregation was probably real, because two independent centromere-linked markers (*trp1* and *leu1*) were used to identify sister spores and the probability of both segregating to nonsisters is $<0.05\%$. In the *cep1* mutant (strain D64, *cep1/cep1*), aberrant meiotic events involving CF(*URA3*) oc-

curred at about the same frequencies as observed for pDK243. Five (3.5%) and 7 (5.0%) loss and meiosis II nondisjunction events were observed, respectively, among the 141 tetrads analyzed, but the most dramatic effect was the increase in the frequency of nonsister segregation (21%). This segregation pattern results from the precocious segregation of the CF sister chromatids at meiosis I.

A fundamental difference exists between the segregation of an unpaired CF and the segregation of an endogenous chromosome. The CF has no homolog, and homolog pairing is known to be important for proper segregation of chromosomes in meiosis (29, 46, 85). To determine whether the absence of a homolog contributed to the high frequency of CF(*URA3*) missegregation in *cep1/cep1* diploids, I analyzed meiotic segregation in strains containing paired CFs. The *URA3* marker on CF(*URA3*) was changed to *LEU2* by gene transplacement (see Materials and Methods) to produce CF(*LEU2*). CF(*URA3*) and CF(*LEU2*) differed only by the insertion of the 2.2-kb *LEU2* segment on the short arm. CF(*LEU2*) displays the same mitotic stability as CF(*URA3*) (data not shown). Strains D68 and D69 each contain a single copy of both CF(*URA3*) and CF(*LEU2*). In a normal meiosis, the CFs should pair and segregate away from each other at meiosis I, resulting in a tetrad with two Ura⁺ sister spores and two Leu⁺ sister spores. The *ade3-2p* marker will segregate 4⁺:0⁻. Figure 5B illustrates the outcomes of aberrant CF segregation in meiosis. Since the fragments are nonessential, nullosomes for the fragments are viable. Fragment disomes can be detected by color phenotype (red) and, if heterozygous, by nutritional phenotype (Leu⁺ Ura⁺). Figure 5B also depicts

the outcome of reciprocal recombination occurring between the centromere and the *URA3/ura3::LEU2* loci on the CF short arm.

Table 6 shows the observed CF meiotic segregation patterns for the wild-type and *cep1* diploids. In the wild-type strain (D68), 2:2 segregation of both CFs was observed 97% of the time and only 5 of 52 tetrads contained spores disomic for one CF. By contrast, numerous cases of CF disomy were observed among the meiotic products of the *cep1/cep1* strain (D69), only some of which could be explained by cells entering meiosis with multiple CF copies. Table 7 catalogs the types of meiotic events observed for tetrads segregating a single copy of each CF homolog. For the wild-type strain, meiosis I and meiosis II nondisjunction events were observed at a frequency of around 5%. The *cep1* mutant displayed no increase in the frequency of meiosis I nondisjunction, but moderate increases in meiosis II nondisjunction (14.5% vs. 3.9%) and loss (1.8% vs 0%) and a large increase in precocious sister segregation at meiosis I (23.6% vs 0%) were observed. In addition, 10.9% of the *cep1* tetrads had undergone multiple missegregation events, the nature of which could not be unambiguously determined. Segregation errors were observed with equal frequency for each CF, and comparing the results for strains D63 (CF(*URA3*)) and D68 (CF(*URA3*)/CF(*LEU2*)), there was no significant difference in the frequency or type of missegregation events observed. [The apparent reduction in precocious sister segregation of CF(*URA3*), 10.9% vs. 20.8%, is not statistically significant by chi-square test ($P > 0.10$).] The presence or absence of a homolog appeared to have little influence on the meiotic segregation of the CFs in *cep1* mutants.

TABLE 6
Meiotic segregation of paired CFs^a

A. Strain D68 (<i>CEP1/CEP1</i>)							
	CF(<i>URA3</i>)					Total	
	4:0	3:1	2:2	1:3	0:4		
CF(<i>LEU2</i>)	4:0	0	0	0	0	0	0
	3:1	0	0	0	0	0	0
	2:2	0	0	49 (3)	2 (2)	0	51
	1:3	0	0	0	0	0	0
	0:4	0	0	1	0	0	1
	Total	0	0	50	2	0	52 (5)
B. Strain D69 (<i>cep1/cep1</i>)							
	CF(<i>URA3</i>)					Total	
	4:0	3:1	2:2	1:3	0:4		
CF(<i>LEU2</i>)	4:0	0	0	1 (1)	0	1	2
	3:1	0	0	0	4 (1)	1 (1)	5
	2:2	0	1 (1)	38(15)	6 (3)	5	50
	1:3	1 (1)	2 (1)	5 (1)	2 (1)	1	11
	0:4	0	0	4 (2)	0	0	4
	Total	1	3	48	12	8	72 (28)

^aData are for four viable spore tetrads. For each class, the number of tetrads containing one or more CF disome is given in parentheses (includes disomes arising through premeiotic events).

TABLE 7
Types of aberrant CF segregation^a

Event	No. of tetrads (%)	
	<i>CEP1</i>	<i>cep1</i>
Proper segregation (4:0)		
Non-recombinant	41 (80.4)	20 (36.4)
Recombinant ^b	5 (9.8)	7 (12.7)
Aberrant segregation		
Meiosis I nondisjunction	3 (5.9)	4 (7.8)
Meiosis II nondisjunction		
CF(<i>LEU2</i>)	0	4 ^c (7.8)
CF(<i>URA3</i>)	2 (3.9)	4 ^c (7.8)
Precocious sister segregation(PSS)		
CF(<i>LEU2</i>)	0	7 ^c (12.7)
CF(<i>URA3</i>)	0	6 ^c (10.9)
Loss	0	1 ^d (1.8)
Other	0	6 ^e (10.9)
Total tetrads scored	51	55

^aData are for tetrads containing 4 viable spores and segregating one copy of each CF.

^bInferred (see text). Scored only in *CEN-LEU2* and *CEN-URA3* intervals.

^cIn four tetrads, both precocious sister segregation and meiosis II nondisjunction occurred. In the table, these events are recorded separately and are thus counted twice.

^dCF(*URA3*)

^eMultiple missegregations for which events could not be unambiguously determined.

Reciprocal recombination is often used as an indicator of functional homolog pairing at the first meiotic division (101). CF(*URA3*) and CF(*LEU2*) differ by only one genetic marker; therefore, it is not possible to definitively monitor recombination between them; however, about 10% of the tetrads from both wild-type and *cep1* diploids yielded what appeared to be the products of reciprocal recombination between these markers and their respective centromeres. As shown in Figure 5, reciprocal recombination between the centromere and the markers on the short arm followed by meiosis I disjunction yields a tetrad in which both markers segregate at the second division. The identical segregation pattern would result from simultaneous precocious sister segregation of both CFs, but this event would be extremely rare in the wild-type strain where precocious sister segregation of a single CF occurs less than 1% of the time and still relatively infrequent (about 1%) in a *cep1* mutant where the precocious segregation frequency is about 10% per CF. We conclude that despite the short physical distance separating the CF centromere and the *LEU2/URA3* loci (about 2 kbp), reciprocal recombination is occurring in this interval and therefore that at least a fraction of the CFs are functionally paired at meiosis I.

Segregation of an unpaired chromosome I. The fact that *cep1* gene disruption affected the meiotic segregation of CF(*URA3*), unpaired or paired, suggested that CP1 was required for the accurate segregation of chromosomes in general. In order to verify this finding for a *bona fide* yeast chromosome, we analyzed the segregation of chromosome I. Since pairing did not appear to significantly influence results for CF(*URA3*), chromosome I segregation was tested in a 2N+1 aneuploid. Segregation of the odd chromosome in a

2N+1 diploid is analogous to the segregation of an unpaired CF; the extra chromosome segregates to sister spores at meiosis II. The result is a tetrad with two sister haploid spores and two sister disomic spores. Precocious segregation at meiosis I results in nonsister-spored disomes. Chromosome *I* is the smallest yeast chromosome (220 kbp), similar in size to the CFs.

Strains D92 and D93 are congenic diploids trisomic for chromosome *I* (Table 1). Each chromosome *I* homolog is marked (*ade1::LEU2/ade1::HIS3/ADE1*), so the disomic products of meiosis can be identified easily by nutritional phenotype (Leu^+His^+ , Leu^+Ade^+ , His^+Ade^+). The results of segregation analysis are given in Table 5. In neither case, was spore viability significantly different than the wild-type or *cep1* average. No aberrant segregation of chromosome *I* was detected for the wild-type strain; disomes were always found in sister spores. In the case of the *cep1/cep1* diploid (D93), 7 of 92 (7.6%) four-viable spore tetrads contained disomes in nonsister spores. This increase in nonsister segregation was statistically significant (chi-square test, $P < 0.05$). Qualitatively, *cep1* gene disruption had the same effect on the unpaired chromosome *I* as it did on the unpaired CF, and quantitatively, the effect was almost as great.

Spore viability. In carrying out the meiotic analyses described above, a significant difference was observed between the spore viability of wild-type and *cep1* tetrads. Table 8 shows the spore viabilities of various diploids used in this study [Strains D92 and D93 are not included, because they are not isogenic with the others. However, their viabilities were not statistically different from the wild type and *cep1* averages.] Overall, wild-type spores were 95.0% viable and *cep1* spores 73.4% viable. The difference was quite

TABLE 8
Spore viability

Diploid		Tetrad Classes (Viable:Inviable)					Total	%Viable
		4:0	3:1	2:2	1:3	0:4		
D24 (<i>CEP1/CEP1</i>)	Obs.	142	17	2	0	0	161	96.7
	Exp. ^a	141	19	1	0	0		
D30 (<i>CEP1/cep1</i>)	Obs.	34	5	0	0	0	39	96.8
	Exp.	34	5	0	0	0		
D25 (<i>cep1/cep1</i>)	Obs.	123	69	57	23	7	279	74.9
	Exp.	88	118	59	13	1		
D38 (<i>CEP1/CEP1</i>)	Obs.	32	3	1	0	0	36	96.5
	Exp.	31	5	0	0	0		
D40 (<i>cep1/cep1</i>)	Obs.	163	93	80	25	18	379	73.6
	Exp.	111	160	86	20	2		
D63 (<i>CEP1/CEP1</i>)	Obs.	110	15	8	2	1	136	92.5
	Exp.	100	32	4	0	0		
D62 (<i>CEP1/cep1</i>)	Obs.	25	2	0	0	0	27	98.2
	Exp.	25	2	0	0	0		
D64 (<i>cep1/cep1</i>)	Obs.	142	73	75	42	11	343	71.4
	Exp.	89	143	86	23	2		
D68 (<i>CEP1/CEP1</i>)	Obs.	52	6	3	0	0	61	95.1
	Exp.	50	10	1	0	0		
D69(<i>cep1/cep1</i>)	Obs.	72	38	36	14	3	163	74.9
	Exp.	51	69	35	8	1		
Totals								
<i>CEP1/CEP1</i>		336	41	14	2	1	394	95.0
	%	85.3	10.4	3.6	0.5	0.3		
<i>cep1/cep1</i>		500	273	248	104	39	1164	73.4
	%	43.0	23.5	21.3	8.9	3.4		

^aThe expected number of tetrads in each class (N_i) is obtained by expanding the polynomial

$$N = T(v + (1-v))^4$$

where T is the total number of tetrads and v is the average viability.

consistent day to day and between strains, indicating that the reduced viability was due to the *cep1* mutation and not to a second unrelated lesion arising by chance in the original *cep1* null mutant or to some nonsystematic variation in sporulation, dissection, or germination conditions. The spore viability of a *cep1* homozygote was rescued to near wild-type levels (90.1%) by a CEN plasmid carrying the *CEP1* gene (data not shown), and spores obtained from *cep1* heterozygotes (D30, D62) displayed wild-type viability (Table 8), indicating that the *cep1* mutation was recessive with respect to spore viability. Microscopic examination of the germinated dissection slabs of both wild type and *cep1* strains revealed that of the spores which failed to grow most did not germinate and those that did germinate (approximately 15%) stopped dividing after 2-4 generations.

The pattern of *cep1* spore inviability was not random. Table 8 shows the viability patterns we observed as well as the patterns to be expected if spore death were random. As for overall viability, the distribution of tetrads within the various viability classes (*i.e.*, 4 viable:0 inviable, 3 viable:1 inviable, *etc.*) was quite consistent day to day and strain to strain. Spore death in the wild-type tetrads appeared to be random; however, for all *cep1* strains the observed distribution was different from that which would have been predicted for random spore death. The differences were statistically significant in all cases (chi-square test, $P < 0.001$). (Only tetrads containing four-viable spores were scored so the nonrandomness worked to our benefit since there were many more *cep1* tetrads with 4 viable spores than would be predicted given the overall *cep1* spore viability of 73.4%.)

An average meiotic missegregation frequency of about 3% per chromosome pair (not greatly different from that observed for an unpaired chromosome *I*) would account for the general viability pattern observed, *i.e.*, 57% of *cep1* tetrads contain at least one dead spore. More precisely, viability would depend on the frequency and type of missegregation event occurring. For example, meiosis I nondisjunction of a single chromosome would result in two dead spores per tetrad. Precocious sister segregation or meiosis II nondisjunction would produce tetrads with one inviable spore. In all three cases, one or two disomic spores per tetrad would also be produced. If multiple missegregation events were to occur, complex segregation patterns and greater spore lethality would result, but the probability of aneuploidy in the few surviving spores would be increased.

To survey for the generation of aneuploidy, diploid strains were constructed which would allow ready detection of disomes among the dissected meiotic products. Four chromosomes were marked, chromosomes *III*, *IV*, *V*, and *VII* (see Materials and Methods). In 885 *cep1* tetrads (strains D40, D69, D64), no disomic spore colonies were observed for chromosomes *IV*, *V*, or *VII*. Eight tetrads were found containing one or two chromosome *III* disomes; however, these were all obtained from CF-containing strains and are most likely due to meiosis I nondisjunction events caused by the CF (see Discussion). No chromosome *III* disomes were found among the 379 D40 tetrads. If the decreased *cep1* spore viability were due solely to random chromosome missegregation, we should have detected many disomes. Consider strain D40. Of the 379 tetrads dissected, 216 tetrads having one or more inviable spores were obtained. Assuming the wild-type (D38) spore viability

(96.5%), only 50 of the 379 should have contained one or more inviable spores $[(1-(0.965)^4) \times 379]$; therefore, 166 would have contained a dead spore because of chromosome missegregation and therefore should also have contained a disome. Since we monitored 4 of the 16 chromosomes, about 40 disomes should have been obtained assuming that the missegregation event(s) involved all chromosomes at equal frequency.

Disome detection by electrophoretic karyotyping. Considering the frequencies at which the CFs and chromosome *I* missegregated, it was surprising that no disomic strains were detected in the genetic screen. As an additional screen for monitoring all of the chromosomes simultaneously, the electrophoretic karyotypes of *cep1* segregants from tetrads containing inviable spores was analyzed. It was expected that for chromosomes separated in agarose gels, those which were carried in greater than single copy would be visually identifiable in that they would stain more intensely than single copy chromosomes. A total of 50 strains representing 26 tetrads that had at least one inviable spore were analyzed. Of these 26 tetrads 8 contained three viable spores, 8 contained 2 viable spores, and 10 contained a single viable spore. For all of these strains 11 chromosomes were well resolved in the gels. Figure 6 shows a representative ethidium bromide stained gel which contains DNA samples from strains suspected of carrying an extra chromosome. Among the 50 strains analyzed, six appeared to be disomic for a single chromosome. In order to determine if the suspected chromosomes actually contained twice as much DNA as monosomic chromosomes, the amount of DNA in the chromosome bands was quantitated by densitometric analysis of photographic negatives of the gels. The results, shown in Table 9, indicated that all of the

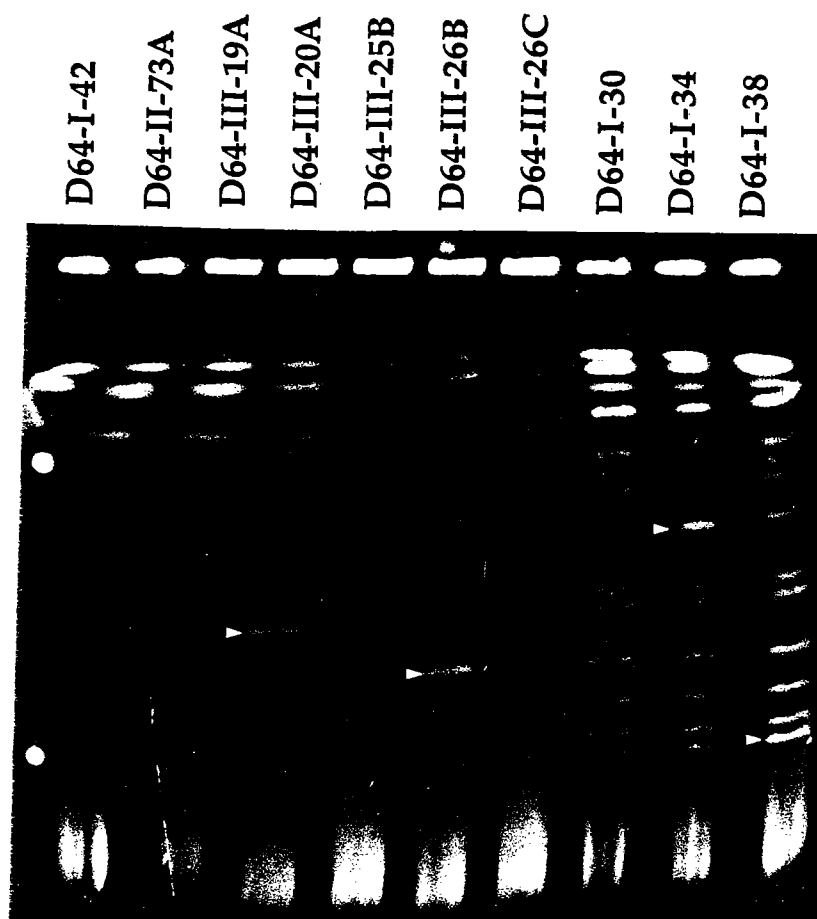


Figure 6. Electrophoretic karyotype of D64 segregants from tetrads with inviable spores. The I, II, and III in the strain numbers indicate tetrads contained 1, 2, or 3 viable spores, respectively. Yeast chromosomal DNA was prepared as described in Materials and Methods and chromosomes were separated in 1% agarose gels by CHEF electrophoresis. Chromosomes suspected as being disomic by virtue of their particularly high staining intensities are indicated by arrowheads.

TABLE 9

Relative staining intensities of electrophoretically separated chromosomes^a

Chromosome	kb	Strain ^b					
		III-20A	III-26B	II-74B	I-34	I-38	I-42
<i>I</i>	220	-	0.6	1.2	-	1.8	1.2
<i>VI</i>	280	-	0.6	1.2	-	1.1	0.9
<i>III</i>	360	-	0.5	2.1	1.1	1.0	1.9
<i>IX</i>	445	1.0	1.0	1.0	1.0	1.0	1.0
<i>VIII</i>	555	2.1	-	-	-	-	-
<i>XI</i>	690	0.8	-	-	1.8	0.8	1.0
<i>X</i>	760	-	-	-	0.9	0.9	0.9
<i>XIV</i>	800	-	-	-	0.9	0.8	0.9

^aFor each strain, densitometry scan values for chromosome band staining intensities were normalized to chromosome *IX* and corrected for chromosome size. Chromosomes suspected of being disomic by visual inspection are highlighted in bold.

^bAll are D64 segregants; I, II, and III indicate strains were from tetrads with 1, 2, and 3 viable spores, respectively.

visually identified disomes had a staining intensity that was roughly twice that of neighboring chromosomes. As additional verification, all of the strains identified as carrying a disomic chromosome were analyzed genetically in crosses to tester strains which carry markers on all sixteen chromosomes. In all six cases, trisomic segregation of the suspected chromosomes was observed which is indicative of disomy in the parent strain (not shown). These results indicate that the sensitivity of endogenous chromosomes to CP1 deficiency is not limited to chromosome *I*. In addition, for the two tetrads with three viable spores, the disomic strains (chromosomes *VIII* and *IX*) were nonsisters to the inviable spores which is consistent with their having arisen due to precocious sister segregation events.

CHAPTER V

MUTATIONAL ANALYSIS OF THE *SACCHAROMYCES CEREVISIAE* CENTROMERE-BINDING PROTEIN CP1

CP1 plays an important role in two apparently unrelated cellular processes, chromosome segregation and methionine metabolism. At centromeres, CDEI-bound CP1 facilitates kinetochore assembly and/or function [(5), Chapter IV]. The specific requirement of CP1 in methionine biosynthesis is not clearly understood, but all sequenced methionine biosynthetic (*met*) genes contain CDEI sites in their promoter regions, and CP1 appears to be involved in the regulation of expression of at least two of these genes (97, 98). In undertaking a mutational analysis of CP1, I addressed three major questions concerning CP1 function: Is DNA binding essential? Is DNA binding sufficient? Can the chromosome segregation and gene expression functions of CP1 be separated by mutation?

Site-directed *CEP1* mutant alleles. The site-directed *cep1* mutants constructed for this study are shown in Figure 7. From preliminary work and published results (64), it was known that N-terminal truncations of CP1 removing up to 210 codons retained full function *in vivo*; therefore, mutations were targeted to the b-HLH and C-terminal domains of the protein. The LI-alleles contain in-frame linker insertions at naturally occurring *SspI* (LI-259), *PstI* (LI-301), and *MluI* (LI-318PG, LI-318SR) restriction sites. CA302, actually an internal deletion of 31 codons, is effectively a C-terminal truncation allele, because translation is terminated by multiple nonsense codons at the codon 301 deletion endpoint. R235H is a point mutant obtained by hydroxylamine mutagenesis; it results in amino acid 235 being changed from arginine to histidine.

TFEB is a mammalian b-HLH factor that has a binding specificity similar to CP1 (18). A segment of TFEB cDNA encoding the DNA-binding

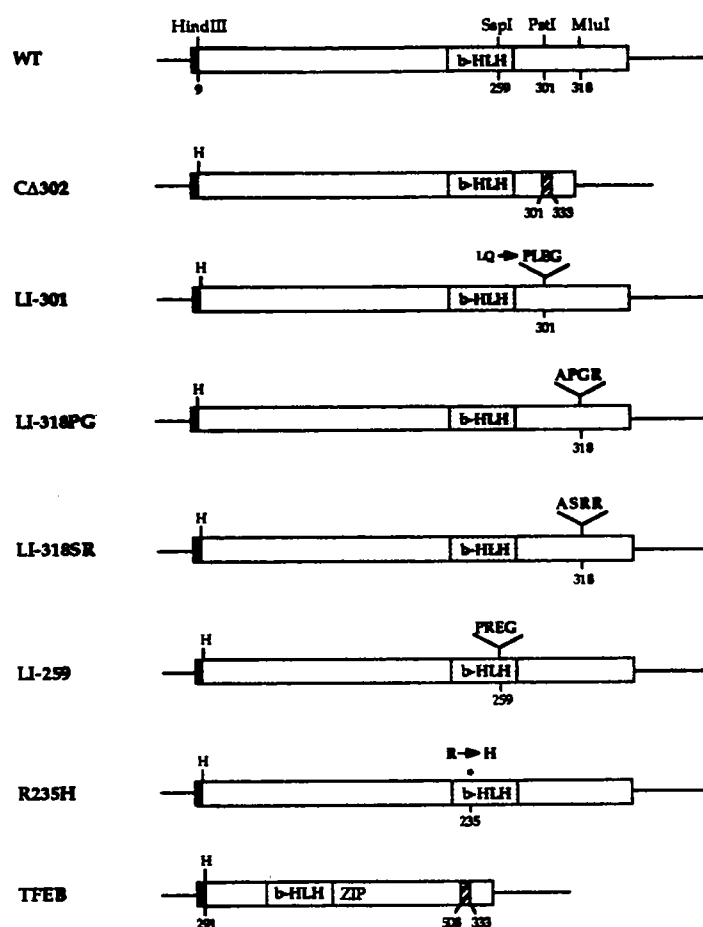


Figure 7. *CEP1* mutant alleles. Alleles were constructed as described in Materials and Methods. Numbers below the diagrams indicate codon positions. The b-HLH domain of CP1 extends from codon 220 to codon 272. LI-301 is a linker insertion at the PstI site which replaces codons 302 and 303 (LQ) with those indicated. The remaining linker insertions contain in-frame insertions of four codons immediately 3' to the codon positions indicated. The mammalian TFEB allele contains a portion of a TFEB cDNA (18) linked by an XbaI nonsense linker to *CEP1* 3'-sequences (codon 333). The approximate positions of the heterologous b-HLH domain and the leucine zipper (ZIP) of TFEB are indicated.

domain was cloned into CP1 expression vectors to determine if a heterologous CDEI-binding protein could confer CP1 function. All of the alleles, including the wild-type, encode an epitope tag (FLAG) at the protein N-termini but retain native *CEP1* promoter and translation initiation sequences. Substituting the eight amino acid FLAG for the natural N-terminus leads to no measurable alteration in CP1 function (data not shown). The results of CP1 functional assays are given in Table 10. NΔ208 was included as an example of a functional N-terminal truncation. This allele lacks amino acids 1-208. NΔ333 was included as a negative control; it retains only codons 333-351 (see Figure 10).

The R235H and LI-259 alleles which affect the basic and HLH regions, respectively, yielded no detectable DNA binding activity when expressed in *E. coli* and displayed no *cep1*-complementing activity when expressed in yeast. These results are completely consistent with numerous findings demonstrating that for this class of proteins the b-HLH domains are essential for DNA binding (25, 94, 102). The TFEB allele was also incapable of conferring detectable CP1 function in yeast; however, in this case the protein was fully capable of binding DNA. *In vitro*, the apparent CDEI-binding affinity of TFEB was equal to that of the fully functional NΔ208 protein, and only 2-fold lower than that of wild-type CP1. Apparently, CDEI-binding activity alone is insufficient to confer CP1 function *in vivo*. (Evidence that TFEB is expressed and binds DNA *in-vivo* is presented below.)

Among the C-terminal mutations, LI-301 and CA302 affect a region, amino acids 292-320, which has been shown to be essential for dimerization and DNA binding (27). Neither mutant protein was able to rescue *cep1*

TABLE 10
Phenotypes Conferred by *CEP1* Alleles

Allele	Methionine Prototrophy		CFVII Loss Rate ^a		CDEI Binding ^d
	YCp	YEp	Absolute ^b	Relative ^c	
WT	6	6	3.0x10 ⁻⁴	1.0	1.0
NA333 (30°)	0	0	1.2x10 ⁻²	37	<0.002
NA333 (22°)	0	0	1.1x10 ⁻²	40	
NA208	6	6	1.4x10 ⁻⁴	0.5	0.5
CA302	0	0	1.1x10 ⁻²	37	0.016
LI-301	0	0	9.3x10 ⁻³	31	0.024
LI-318PG (30°)	1	6	5.0x10 ⁻³	17	0.12
LI-318PG (22°)	3	6	6.2x10 ⁻³	21	
LI-318SR	6	6	3.2x10 ⁻⁴	1.1	0.14
LI-259	0	0	1.0x10 ⁻²	33	<0.002
R235H	0	0	9.3x10 ⁻³	31	<0.002
TFEB	0	0	9.0x10 ⁻³	30	0.5

^aDetermined by fluctuation analysis using YCp plasmid transformants.

^bLoss events/cell/division.

^cRelative to WT.

^dRelative apparent binding affinities determined using *E. coli* extracts.

phenotypes, even when expressed from multicopy vectors. Although dimerization of CP1 was demonstrated to be required for DNA binding (64), both gene products retained some CDEI-binding activity. Since CA302 lacks virtually all of the dimerization domain identified by Dowell et al. (27), the HLH domain itself probably mediates the CP1 dimerization required for CDEI site recognition. While the 50-fold or so reduced CDEI-binding affinity of the LI-301 and CA302 gene products emphasizes the importance of this protein domain for DNA binding, it is possible that the reduced CDEI binding affinity alone may not account for the observed lack of function, at least with respect to chromosome segregation. It has been shown that a centromere CDEI mutation which reduces CP1 binding affinity 35-fold does not affect mitotic chromosome loss rate (3, 36).

Mutations LI-318SR and LI-318PG resulted in either no or partial loss of function *in vivo*, although both mutations reduced CDEI-binding activity. A single copy of the LI-318SR allele completely rescued CP1 functions to wild-type levels despite a 9-fold-reduced apparent binding affinity. LI-318PG has a similarly reduced DNA binding activity. This mutation, however, was less capable of conferring CP1 function. When carried on a single-copy (YCp) vector, LI-318PG conferred a temperature-sensitive Met phenotype and a partially defective chromosome loss (Chl) phenotype. Expressed from a multicopy (YE_p) vector, LI-318PG gave complete rescue of the Met phenotype and nearly complete rescue of the Chl phenotype (loss rate of 8.3×10^{-4} loss events/division). One explanation for this result was that the LI-318PG gene product was functional but that intracellular levels were low, possibly due to

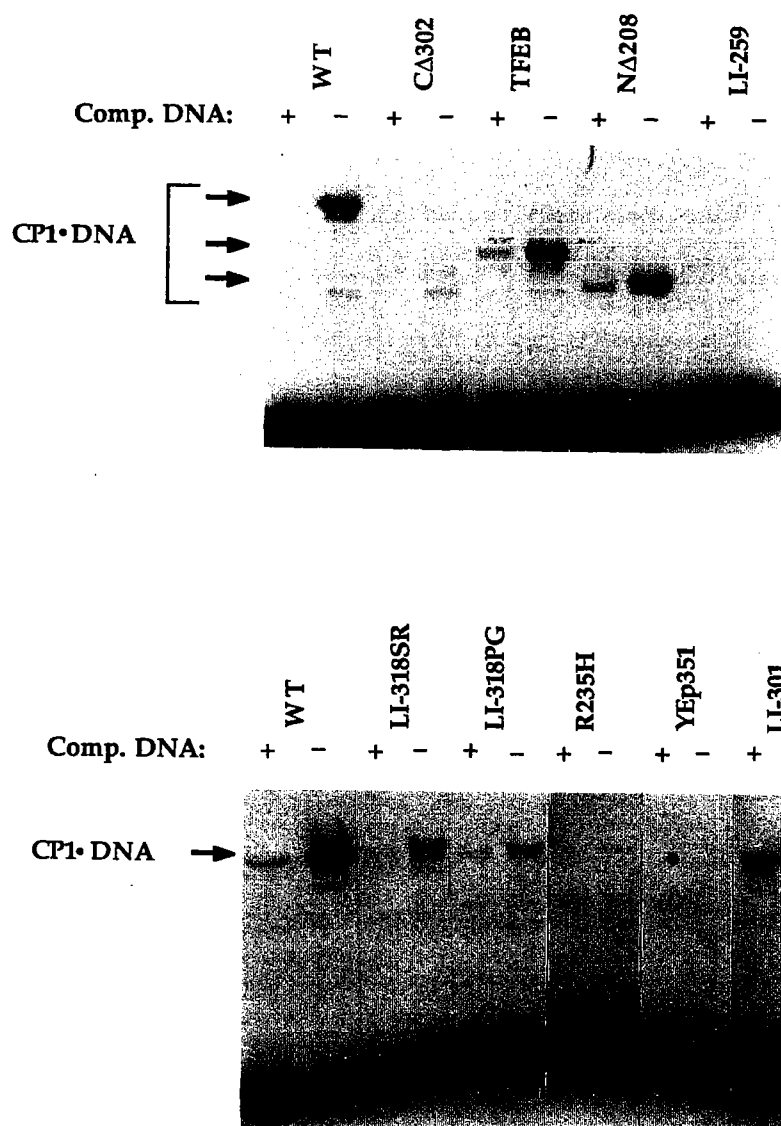


Figure 8. DNA binding activity of mutant *CEP1* alleles in yeast. Gel shift DNA binding assays were carried out as described in Materials and Methods. The assay shown utilized extracts (20 μ g of total protein prepared from *cep1* transformants carrying the mutant alleles on multicopy (YEp) plasmids. Competitor DNA was 2.5 μ g of specific (+) or non-specific (-) plasmid DNA per reaction.

decreased protein stability. Together, these results suggest that normal levels of CP1 in the cell are in functional excess.

DNA binding activity of the mutant CP1 gene products in yeast was analyzed by DNA binding assays of crude extracts (Figure 8). Since equal amounts of crude protein were used for the binding assays, the amount of specifically bound probe in each reaction should have reflected both CDEI-binding affinity and the relative concentration of the mutant protein in the extract. For the most part, the observed binding activity corresponded to what was expected given the estimated binding affinities of the *E. coli*-produced proteins: wild-type activity was observed for N Δ 208 and TFEB; reduced binding was observed for LI-318SR and LI-318PG; and no binding was detected for R235H and LI-259. In all cases where binding was observed, the activity was specific, as it was inhibited in the presence of excess unlabeled CDEI site-containing DNA. The results for two of the mutant gene products, LI-301 and C Δ 302, did not correspond to expectations. Given the 40- and 60-fold-reduced DNA binding affinities of these proteins, respectively, low binding activity in yeast extracts had been predicted. Instead, no detectable binding was observed for C Δ 302, but nearly wild-type activity was observed for LI-301. In addition, the LI-301 extract produced a significant amount of bound probe in the presence of the CDEI competitor. The source of extracts for the experiment shown in Figure 8 was *cep1* transformants expressing the various alleles on multi-copy plasmids, but the same relative results were obtained when extracts were prepared from transformants carrying single-copy plasmids (not shown).

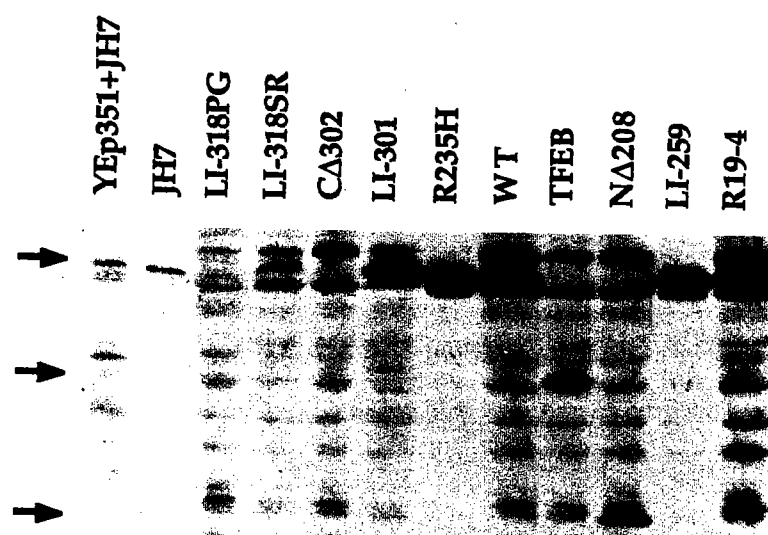


Figure 9. Immunodetection of epitope-tagged proteins. A western blot (see Materials and Methods) was carried out using the same crude yeast extracts that were used in the binding assay shown in Figure 6. Thirty μ g of total protein were loaded except for extracts of strains carrying LI-259 and R235H where only 5 μ g were loaded and strain R19-4 where 80 μ g was used. R19-4 carries a chromosomal copy of *CEP1*^{FLAG}. The first two lanes contain approximately 0.1 ng of *E. coli*-produced CP1 (pJH7) loaded alone (lane 2) or mixed with control *cep1* yeast extract (lane 1).

A direct measure of the amounts of mutant gene products in yeast was obtained by immunoblot analysis using a monoclonal antibody directed against the epitope tag. Figure 9 shows an immunoblot of the extracts used for the binding assays of Figure 8. The NA208, TFEB, and LI-318SR gene products were detected at levels similar to wild-type CP1. The non-binding mutant proteins R235H and LI-259 were expressed at very high levels as was LI-301. The high relative concentration of LI-301 gene product probably explains why appreciable CDEI-binding activity was detected in the yeast extracts despite a significantly reduced DNA binding affinity. In contrast, LI-318PG and CA302 proteins were undetectable by western blotting, even though binding activity was detected for LI-318PG in this same extract. Evidently, the DNA binding assays are more sensitive than immunoblotting. In any event, this result indicates that LI-318PG is present at a significantly lower level than LI-318SR or wild-type CP1. CA302 must be present in even lower amounts, if at all.

CEP1 frame-shift mutants. The LI-318PG results suggested that a very low level of CP1 gene product could support significant CP1 function. Analysis of several *cep1* frame-shift alleles provided additional support for this conclusion. In constructing N-terminal truncation mutants (e.g., NA208), a number of alleles were generated in which the 5'-deleted *CEP1* coding region was out-of-frame relative to the initiator ATG and FLAG element (see Chapter II). Surprisingly, the frame-shifted constructs were able to rescue *cep1* phenotypes to varying degrees (Figure 10). Evidently these alleles were being expressed at some functional level, presumably by downstream translational initiation or re-initiation.

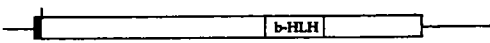
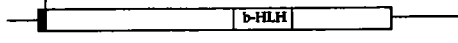
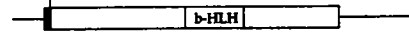

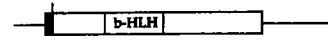
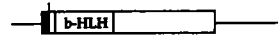
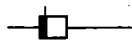
		bp to nearest in-frame ATG	Methionine phenotype	CFVII Loss Rate	
				Absolute	Relative
WT	GAT GAT <u>AAG CTT</u> TCT ACT (0) 	-	6	3.0×10^{-4}	1.0
NΔ33	GAT GAT aag ctt cGG CCA GGA (+1) 	359	1	1.1×10^{-2}	34
NΔ86	GAT GAT aag ctt cGA AGA TGC (-1) 	199	5	9.4×10^{-4}	3
NΔ137	GAT GAT aag ctt cAA CCC CAT (+1) 	47	2	8.0×10^{-3}	27
NΔ162	GAT GAT aag ctt cGC AAT CTA (+1) 	17	4	1.6×10^{-3}	5
NΔ208	GAT GAT aag ctt cAA CCT ACT (0) 	-	6	1.4×10^{-4}	0.5
NΔ332	GAT GAT aag ctt cAC TCA CAA (+1) 	-	0	1.2×10^{-2}	40

Figure 10. Amino terminal *CEP1* truncation alleles and their ability to complement *cep1* phenotypes. The fusion joint at the deletion endpoint is shown above each diagram, HindIII linker sequences in lower case, *FLAG* and *CEP1* sequences in upper case. For the wild type allele (WT), the naturally occurring HindIII site spanning codons 9/10 is underlined. Nucleotides are grouped as triplets to indicate the reading frame established by the native ATG initiator codon. Underlined triplets indicate the native CP1 reading frame with the numbers in parentheses indicating the net shift in reading frame relative to the initiating ATG and FLAG. The alleles are named to indicate the native *CEP1* codon at the fusion site. Methionine phenotype and CFVII loss rates were determined as described in Chapter II. Absolute loss rates are loss events/cell/division.

In all cases, there was a direct and quantitative correlation between the ability of an allele to promote methionine-independent growth and its ability to rescue the *cep1* mitotic chromosome loss defect (Figure 10). The difference in complementation ability may reflect the level at which the frame-shift alleles would be expected to be expressed. With the exception of NΔ86, the shorter the distance between the deletion endpoint and the nearest in-frame ATG (codon 153 for NΔ33, NΔ86, and NΔ137; codon 168 for NΔ162), the better the allele at complementing *cep1* defects. This was interpreted as suggesting that the further downstream the nearest in-frame ATG was to the frameshift (fusion) site, the less efficiently translational reinitiation occurred. NΔ86 differs in that it is a -1 frame shift, while the others are +1 frameshifts. There are three tandem ATG's in the -1 reading frame located in the FLAG codons immediately upstream of the fusion site. Presumably translation of NΔ86 is initiated relatively efficiently at one of these sites, while translation of the +1 frameshift alleles is initiated less efficiently from a downstream ATG.

Figure 11 shows results of *in vitro* DNA binding assays of extracts from yeast expressing the N-terminal truncation alleles. The only extracts in which specific CDEI binding activity was detected were those of strains carrying the wild-type, NΔ208, and NΔ86 alleles. These three alleles were also the best at complementing *cep1* phenotypes. The NΔ208 extract had wild-type binding activity, while an equal amount of NΔ86 extract protein bound markedly less probe, consistent with a reduced expression of this allele due to the frameshift mutation. The NΔ86 complex was of a size consistent with it being initiated from an ATG very near the fusion site (*i.e.*, within FLAG). No CDEI-specific binding activity was detected in extracts made from transformants carrying

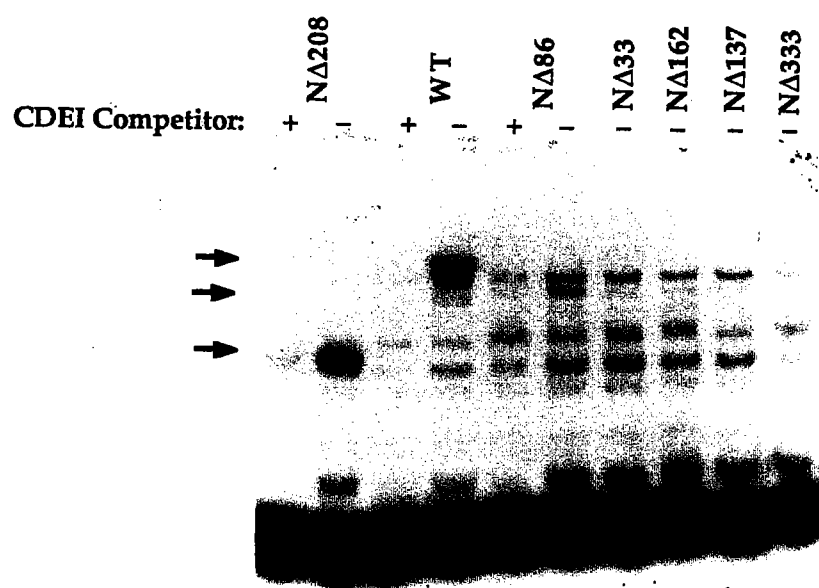


Figure 11 CDEI binding activity of strains expressing N-terminal truncation alleles of *CEP1*. Binding assays were carried out as described in Materials and Methods. Each reaction contained 20 μ g of total protein from extracts made from RB31-3B transformants carrying the *CEP1* alleles on single-copy (YCp) plasmids. Specific (+) or non-specific (-) plasmid competitor DNA was included in each reaction.

the other out-of-frame alleles, suggesting that these alleles were expressed at levels lower than NΔ86. The overall phenotype of strains carrying the frame-shift alleles was the same as that of the LI-318PG transformant, *i.e.*, weakly Met⁺ and intermediate between *cep1* and wild-type with respect to chromosome loss.

Dominant negative effects. Several of the mutants might be expected to exhibit dominant negative phenotypes. They fall into two classes: those that retain CDEI binding activity but, lacking some other CP1 domain or function, fail to complement *cep1* defects (TFEB, LI-301); and those that are unable to bind DNA, but retain the ability to dimerize and/or interact with other protein cofactors (R235H, LI-259). The first class could interfere with wild-type CP1 by competing for CDEI binding sites. The second class might interfere by forming non-DNA binding heterodimers with the wild-type CP1 or by titrating other required components. Somewhat surprisingly, none of the mutant alleles expressed on either single- or multicopy plasmids in a wild-type strain caused any detectable defect in methionine biosynthesis (Figure 12) or in mitotic chromosome segregation (assayed by colony sectoring; not shown).

Dominant negative effects were also tested for in a *cep1 pho80* background. O'Connell and Baker (75) previously showed that *pho80* mutations suppress *cep1* methionine auxotrophy. Suppression appears to be due directly to the action of the positive transcription regulator PHO4. CP1 and PHO4 share DNA binding specificity (24, 31), and the earlier results suggested that PHO4 can bind to CDEI sites in *met* gene promoters and activate transcription (75). This activation pathway is not available under normal growth

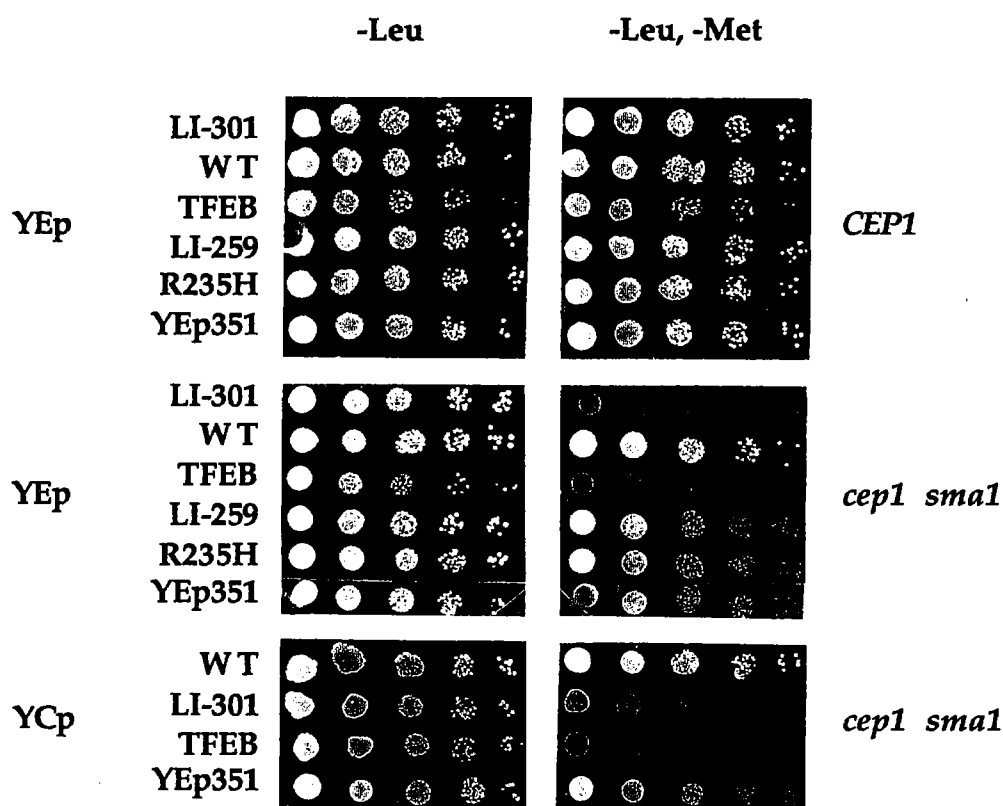


Figure 12. Dominant negative effects of *cep1* mutant alleles on the growth of strains on medium lacking methionine. Cells were grown to mid log phase in medium lacking leucine, centrifuged, and then diluted in medium lacking both leucine and methionine to a final concentration of 2×10^6 cells per ml. For each strain, a series of five, 5-fold serial dilutions were made in the double dropout medium and 5 μ l aliquots were spotted onto plates lacking leucine, and onto plates lacking both leucine and methionine. Plates were incubated at 30° and were photographed after 48 (*CEP1*) or 72 hours (*cep1 sma1*).

conditions, because PHO4 is kept inactive by its negative regulator PHO80 (100, 107). Mutations in *pho80* free PHO4 to act constitutively. Other mutations which lead to constitutive activation of PHO4 (e.g., *PHO4^c*, *pho84*) also suppress *cep1* methionine auxotrophy (75).

Results of an assay testing CP1 mutants for a dominant negative Met phenotype in a *cep1 pho80* (*sma1*) double mutant strain are shown in Figure 12. [The *sma1* (suppressor of *cep1* methionine auxotrophy) complementation group is allelic to *pho80*.] When carried on either single- or multicopy vectors, LI-301 and TFEB caused a dramatic reduction in the ability of the respective strains to grow in the absence of methionine. Both of these proteins fall into the first class of mutants mentioned above--they bind DNA but are otherwise nonfunctional. This result suggests that LI-301, despite its 40-fold reduced CDEI binding affinity, occupies CDEI sites *in vivo*. Neither of the non-DNA binding mutants R235H or LI-259 showed an inhibitory effect on the growth of the *cep1 sma1* strain in the absence of methionine.

To rule out direct inactivation of PHO4 by TFEB and LI-301, *PHO5* expression in the transformants was assayed. *PHO5* is a downstream target of PHO4, and its gene product, the repressible acid phosphatase (rAPase) is readily assayed. In contrast to the ability of TFEB and LI-301 to interfere with PHO4-mediated *MET* gene expression, these mutants were little affected in their expression of *PHO5* (Table 11). In fact, modest increases in rAPase activity were observed, similar to the increase observed for the strain expressing the wild-type *CEP1* allele. All other strains had rAPase activities similar to that measured for the control (YEp351) transformant.

TABLE 11
PHO5 Activity of *sma1* Transformants^a

Allele	YCp	YEp
WT	135 (\pm 5)	120 (\pm 0)
YEp351	ND ^b	87 (\pm 2)
LI-301	150 (\pm 50)	105 (\pm 15)
LI-259	ND ^b	91 (\pm 7)
R235H	ND ^b	83 (\pm 13)
TFEB	180 (\pm 20)	205 (\pm 15)

^amilliunits of rAPase, 1 unit = 1 μ mol PNPP/min., average of two independent transformants, (\pm total deviation).

^bND, not determined.

CHAPTER VI

DISCUSSION

The primary goals of this research were to determine the role that CP1 plays in centromere function and to learn how CP1 functions in that role. The mitotic chromosome-loss phenotype of *cep1* null mutants was shown to be due to nondisjunction of sister chromatids during chromosome segregation. In meiosis, *cep1* strains exhibited increased aberrant chromosome segregation; the predominant event being precocious segregation of sister chromatids during the first meiotic division. Both of these missegregation phenotypes can be explained by the failure of sister chromatids to remain attached until they are due to be segregated, indicating that the role that CP1 plays in centromere function is to aid in the maintenance of sister chromatid cohesion. A mutational analysis of CP1 was undertaken to begin to learn how it functions in this role. Results of that analysis led to the hypothesis that CP1 acts by configuring chromatin in the vicinity of its binding sites into a conformation facilitating the interaction of other DNA-binding proteins.

The genetic analysis of CP1 function was initiated by the isolation of *CEP1*, the gene encoding CP1 (5). The cloning of the gene allowed the construction of strains in which it was disrupted. Haploid cells containing a disrupted *CEP1* gene are viable, indicating that *CEP1* is nonessential. However, *cep1* strains grow more slowly than their isogenic *CEP1* counterparts, exhibit increased rates of mitotic chromosome, CF, and CEN plasmid loss, and are methionine auxotrophs. To construct the *cep1::URA3-11* disruption allele 900 bp of coding region and about 600 bp of 3'-flanking DNA were deleted. A possible explanation for the pleiotropic effects of this mutation would be that the deletion of downstream sequences disrupts a neighboring gene. The experiments described in Chapter III demonstrate that the observed

phenotypes are due solely to the lack of functional CP1. Haploid derivatives of strain R31, which carry the *CEP1* gene disruption that removes only *CEP1* coding sequences, have phenotypes indistinguishable from those of the strain carrying the *cep1::URA3-11* allele--i.e. slow growth, increased CF loss, and methionine auxotrophy. The detailed analysis of the mitotic chromosome loss phenotype indicated that the predominant event which results in cells having lost a chromosomal element is nondisjunction (2:0 segregation). This type of missegregation can be explained either by premature separation of sister chromatids followed by random segregation of the sisters or, as the term connotes, by a failure of the sister chromatids to disjoin and segregate from each other during mitosis.

In order to further examine the role of CP1 in centromere function, the genetic analysis of chromosome segregation during meiosis in *cep1* null mutant strains was undertaken. Previous work indicated that CDEI is required for proper centromere/kinetochore function during meiosis (23, 36). In particular, CEN plasmids containing CDEI-deleted centromeres were found to exhibit high frequencies of precocious sister segregation at meiosis I. The effect was also observed for chromosome *III* containing a CDEI-deleted centromere (23), although the latter result was not confirmed in a subsequent study (36), suggesting that chromosomes are less sensitive to a deficiency of CP1. The results presented in Chapter IV are consistent with these findings. Homozygous *cep1* diploids display significantly increased meiotic missegregation of a CEN plasmid, nonessential chromosome fragments, and native yeast chromosomes. The predominant aberrant event observed in all cases is precocious sister segregation at meiosis I. To be segregated properly during meio-

sis, separation of replicated sister chromatids must be delayed until anaphase of the second division. Precocious sister segregation results when sisters separate prematurely and segregate at the first division. Two possible scenarios are considered: (1) the kinetochore complex "splits" at anaphase I as it normally would at anaphase II or (2) one or both sister chromatids detach from the kinetochore complex and segregate randomly. The latter mechanism is preferable for explaining the precocious sister segregation observed in *cep1/cep1* diploids because a general defect in chromatid-kinetochore adhesion would also explain the increased mitotic and meiosis II nondisjunction observed with *cep1* mutants. In addition, since CP1 specifically binds centromere DNA, it is not hard to imagine that as part of the kinetochore complex CP1 would promote chromatid-kinetochore interaction.

The increased chromosome *I* missegregation in the $2N+1$ *cep1* strain and the detection of strains carrying disomic chromosomes by electrophoretic karyotyping demonstrated that the *cep1* defect extends to native chromosomes as well as plasmids and recombinant CFs. In addition, chromosome *I* also missegregates frequently in a normal *cep1/cep1* diploid. The analysis of 125 tetrads from a diploid strain derived from D93 (*cep1/cep1*) but containing only two chromosome *I* homologs (*ade1::HIS3/ade1::LEU2*) identified two His⁺Leu⁺ segregants (both in tetrads segregating the extra chromosome 2:2) which were subsequently confirmed as chromosome *I* disomes by pulsed field gel electrophoresis. The disomes were found in separate tetrads, so the missegregation frequency was 1.6%. Disomes were also detected among D64 segregants by CHEF gel analysis for chromosomes *I*, *III*, *VIII*, *IX*, and *XI*. Among these six, two were for chromosome *III* and probably resulted from an inap-

appropriate pairing event between the CF and chromosome *III* (see below). Since we have no simple genetic method for assaying for disomy of chromosomes *VIII*, *IX*, and *XI*, the actual frequencies at which these chromosomes missegregate were not determined. Although the overall frequency of disomes detected by this method seems relatively high, taking the 26 tetrads analyzed to be an accurate representation of the 625 tetrads with inviable spores, only 12% of tetrads with dead spores can be definitively concluded to have arisen directly from missegregation of chromosomes.

Both Surosky and Tye (95) and Shero *et al.* (90) have observed increased meiosis I nondisjunction in strains containing chromosome fragments. The nondisjunction occurs when the CF recombines with an endogenous chromosome. In doing so the segregation of the natural homologs is disrupted such that they nondisjoin at meiosis I, resulting in a tetrad with two inviable sister spores lacking the chromosome and two viable disomic sister spores. This type of nondisjunction is specific for the chromosome homologous to the CF and occurs at a frequency of 4-7% depending on the CF (90, 95). I also observe what appears to be CF-dependent meiosis I nondisjunction. In my case, the nondisjunction events involve chromosome *III* and occur at a lower frequency, about 1%. This low level of chromosome nondisjunction does not interfere in the analyses of CF segregation, because only tetrads containing four viable spores were scored.

The viability of *cep1* spores is significantly reduced, and the pattern of inviability is nonrandom. While on the average, 1 in 4 spores is inviable, almost half of the *cep1* tetrads contain 4 viable spores, far more than would be expected (see Table 8). Although the possibility that the inviability is directly

attributable to chromosome missegregation was not ruled out, the results suggest that it is not. If it was, I should have detected many aneuploids among the viable spores from *cep1* meioses in the genetic screen (see p. 57). In addition, although the CHEF gel analysis indicated the some of the inviability was the direct result of chromosome missegregation, the frequency at which it was detected was insufficient to result in the overall reduction in spore viability. The viability defect may be unrelated to the kinetochore function of CP1. Disrupting *CEP1* has pleiotropic effects: generation time is increased by 35% and the strains are methionine auxotrophs (5). The reduced *cep1* spore viability could be due to the loss of some general CP1 function.

It was surprising that chromosome missegregation was not detected in the genetic screen. No aneuploidy was observed for chromosomes *IV*, *V*, or *VII*, and the few chromosome *III* disomes which were observed could be attributed to CF-induced meiosis I nondisjunction. Several explanations are possible for the apparent lack of aneuploids: some disomic chromosomes could be extremely unstable in the *cep1* background and be lost very early during growth of the spore colony; the *cep1* defect may not affect the chromosomes we monitored; missegregation events may not be randomly distributed among all tetrads. While none of these possibilities can be ruled out, a more favorable direct interpretation of the results would be that for these chromosomes the level of missegregation is below detection by tetrad analysis.

Mitotically, *cep1* mutants show about a 10-fold increase in the loss rate of chromosome *III* (5, 17) and a 25-fold increase in the loss rate of CF(*URA3*) (Table 4). It is difficult to quantitate the meiotic effect of *cep1*, but taking the case of the unpaired CF(*URA3*), the increase in precocious sister segregation is

20-fold (based on observing one event with the wild-type strain). Assuming a 10- to 25-fold increase over the wild-type meiotic chromosome nondisjunction frequency of 1×10^{-4} (measured for chromosome V) (91), the estimated *cep1* meiotic chromosome missegregation frequency would be 1 to 3 events per chromosome per thousand meioses. If this an accurate estimate, it is not so surprising that disomes were not detected in the genetic screen.

The high relative abundance of CP1 in the cell (3, 11) and the pleiotropic effect of *CEP1* gene disruption suggests that CP1 has a more general function in addition to its specific role at the centromere. In the case of the methionine auxotrophy phenotype, CP1 appears to play a role in the regulation of expression of genes involved in methionine metabolism. All *met* genes sequenced to date contain CDEI sites in their promoter regions. These sites have been shown to be required for full expression of *MET25*, the gene encoding *O*-acetylserine sulphydrolase (97). Additionally, expression of *MET16*, the gene encoding phosphoadenylylsulfate (PAPS) reductase, was shown to require both CDEI sites in cis and CP1 in trans (98). The mutational analysis of CP1 described in Chapter V was undertaken to learn more about how CP1 functions in its dual roles in aiding sister chromatid attachment and in regulating *met* gene expression, and to see if these functions could be separated. This analysis also addressed the questions of whether or not DNA binding by CP1 is essential or sufficient for CP1 function.

CP1 belongs to the family of b-HLH DNA binding proteins (17) which recognize the core consensus E-box element CANNTG (73). Specific DNA recognition by b-HLH proteins is mediated by the basic region of the b-HLH domain, while the HLH subdomain directs protein dimerization which is re-

quired to bring basic region residues into the proper configuration for DNA contact (53). Two of the CP1 mutations are located in the b-HLH domain and reduce specific CDEI-binding activity by at least 500-fold. The R235H point mutation changes a conserved arginine residue in the basic region which has been shown to be responsible for specific recognition of the inner dinucleotide of the consensus CANNTG binding site motif (CACRTG in the case of CP1) (24, 99). The LI-259 mutation disrupts the second helix of the HLH domain and would be expected to affect dimerization. Neither of these non-DNA binding mutants retains any measurable function *in vivo*, although both are present at greater than wild-type levels.

Dowell *et al.* (27) have reported that the C-terminal region of CP1 (spanning codons 292-320) comprises a primary and essential determinant for CP1 dimerization and that this domain is essential for all CP1 functions. My results support the conclusion that this region is critical for overall CP1 function; however, the CA302 allele lacks two thirds of these amino acids but still binds DNA specifically indicating that the HLH domain of CP1 is itself sufficient to mediate dimerization. Nonetheless, the significantly decreased DNA binding affinities of the CA302, LI-301, LI-318PG, and LI-318SR proteins confirm the overall importance of this domain for DNA binding. The experiments using these proteins do not address dimerization directly, but it is likely, based on the results of Dowell *et al.*, that these mutations impair DNA binding by reducing dimer stability. In addition to affecting DNA binding, mutations in the C-terminal domain also appear to influence protein stability. The cellular concentrations of LI-318SR and LI-318PG in yeast are signifi-

cantly lower than that of wild-type CP1, while the CA302 mutant protein is undetectable.

Estimates of the cellular concentration of CP1 indicate that it is a relatively abundant protein (3, 11). My results demonstrate that the nuclear concentration of CP1 is, in fact, well in excess of the minimum required to maintain a wild-type phenotype (at least with respect to the two phenotypes analyzed). First, the LI-318SR allele in single copy is wild-type in function even though its apparent CDEI binding affinity is reduced 9-fold. Second, the N-terminal truncation/frame shift alleles, which appear to be maintained at levels lower than wild-type, still provide significant CP1 function. In some cases (NΔ162, NΔ137, and NΔ33), expression levels are so low that no CDEI-binding activity is detectable in cell extracts. In making this argument, an assumption is made that the translation products of the frame-shift alleles have essentially wild-type binding affinity and are stable in the crude extracts (and would have been detected). This is believed to be the case, because in-frame truncation alleles, NΔ208 and NΔ80, NΔ182, and NΔ192 (not shown) all confer full, wild-type Met prototrophy and bind CDEI with approximately wild-type affinity.

While certain mutants (N-terminal truncation/frame-shifts, LI-318PG) restored methionine prototrophy without rescuing the chromosome loss defect (Met⁺Chl⁻), no mutations causing the reverse phenotype (Met⁻Chl⁺) were obtained. Nevertheless, none of the mutations analyzed completely separated the segregational and transcriptional functions of CP1. All mutations having a measurable phenotypic effect invariably affected both functions. For the N-terminal truncation/frameshift series, a direct and quantitative correla-

tion was observed in the degree to which Chl and Met phenotypes were complemented. These data are consistent with CP1 performing a similar function at *met* gene promoters and centromeres.

Mellor *et al.* (65) have proposed that CP1-CDEI-binding is required for the centromere activity of CP1 but not for support of methionine prototrophy. This would imply that CP1 acts via different mechanisms to carry out its multiple functions. Their conclusion is based on the analysis of a site-directed basic region *cep1(cpf1)* mutant, YAG214, which was found to possess no detectable CDEI-binding activity *in vitro*, but which conferred methionine prototrophy and partially rescued centromere function when expressed in yeast. This same phenotype was observed with the NΔ162, NΔ137, and NΔ33 frame-shift alleles: strains grow in the absence of methionine, show intermediate chromosome loss rates, and yield crude extracts devoid of detectable CDEI-binding activity. Since there is no reason *a priori* to predict that these gene products would lack DNA binding activity, the results can be simply interpreted as indicating that less CP1 is required to maintain methionine prototrophy than to maintain optimal centromere function. The Met⁺Chl⁻ phenotype of the YAG214 mutant could be explained similarly. The YAG214 gene product might have a very low but nonzero DNA binding affinity, undetectable by *in vitro* assay but sufficient for partial function *in vivo*.

Convincing evidence exists indicating that CP1 function requires specific DNA binding. The equivalent and nonadditive effect of cis and trans CDEI/*cep1* mutations on mitotic centromere function indicates that CP1 acts through centromeric CDEI sites (5). Domain swap experiments indicate that CDEI-binding specificity is also essential for CP1 to function in activating *met*

gene transcription. Dang *et al.* (24) engineered a hybrid CP1 protein in which the CP1 DNA recognition domain (basic region) was replaced by that of the mammalian b-HLH protein AP4. The resulting mutant, CP1-AP4 had the DNA binding specificity of AP4 (CAGCTG), was unable to bind CDEI sites (CACGTG), and did not confer methionine prototrophy when expressed in yeast. When Met⁺ revertants of CP1-AP4 were selected, all contained an amino acid change in the basic region (amino acid 235) that also reverted the binding specificity to CACGTG.

Although CDEI binding activity appears to be necessary for CP1 function, DNA binding *per se* is not sufficient. The TFEB protein has an apparent CDEI binding affinity close to that of wild-type CP1 and higher than the fully functional LI-318SR protein but is phenotypically inactive. Also, the inactivity of LI-301 is unlikely to be explained solely by its 40-fold-reduced CDEI binding affinity. It has been shown that a centromeric CDEI mutation which reduces CP1 binding affinity 35-fold has no measurable effect on CEN plasmid or chromosome stability (3, 36). Furthermore, strains expressing LI-301 from a multicopy vector are phenotypically *cep1*⁻ but contain wild-type levels of CDEI-binding activity. Both TFEB and LI-301 apparently enter the nucleus and occupy CDEI sites, because both gene products exert a strong dominant negative effect on the ability of PHO4 to activate *met* genes.

Surprisingly, no trans-dominant effects were observed when the *cep1* mutants were overexpressed in a *CEP1* genetic background. There are several examples of dominant negative interactions among b-HLH proteins. The mammalian protein Id is a naturally-occurring inhibitor of the myogenic factor MyoD (7). Id contains an HLH domain capable of interacting with the

MyoD HLH domain but lacks an associated basic region for DNA interaction. MyoD-Id heterodimers are unable to bind MyoD sites (7). In *Drosophila*, the *extramacrochaetae* gene product antagonizes transactivation of proneural genes in an analogous manner by inactivating b-HLH transactivators encoded by the *achaete-scute* complex (28, 34). By the same mechanism, a basic region mutation in the b-HLH factor E-47 has been shown to cause a trans-dominant effect on the DNA binding of both wild-type E-47 and the heterologous protein E-12 (102). CP1 R235H is analogous to the E-47 mutant, but in the case of R235H no dominant negative phenotype is observed. This is consistent with the conclusion that CP1 levels are in excess. If this is the case, it would require extreme overexpression of R235H to reduce the pool of wild-type CP1 homodimers below the critical level. Also, it is possible (even probable) that an equilibrium situation does not exist. As a result of stable DNA binding, CP1 homodimers could be "trapped" in the nucleus, effectively removing them from the soluble pool. Of course, the data do not rule out the possibility that the R235H mutation inhibits dimerization and/or nuclear localization, or that CP1-R235H heterodimers are much less stable than CP1 homodimers.

CP1 has been grouped with RAP1, REB1, and ABF1, proteins which comprise a family of *S. cerevisiae* factors known as general regulatory factors (GRFs) (14, 15, 19, 26, 54). All of these factors are relatively abundant, sequence-specific DNA binding proteins that have many genomic sites of interaction and are involved in diverse chromosomal functions. In addition to having both positive and negative effects on gene transcription, GRFs have been shown to be associated with chromosomal origins of replication (ABF1),

telomeres (RAP1, REB1), and centromeres (CP1, REB1). REB1 (also known as factor Y or GRF2) is a strong positioner of nucleosomes (30).

I propose that the primary function of CDEI-bound CP1 is to configure chromatin into a conformation that facilitates the assembly of both functional kinetochores and active transcription complexes. This model is essentially that proposed for other general regulatory factors like RAP1 and REB1 (14, 19, 26). CP1 is known to influence chromatin structure at gene promoter regions (64). It is not yet known if the CP1-associated chromatin structures are established independently by CP1 or require the participation of cofactors, which could differ in each case. However, CDEI site occupancy alone appears to be insufficient to mediate CP1 function, suggesting that CP1 contains an additional functional domain which interacts with chromatin components. This domain, which apparently lies in the C-terminal third of the protein, may directly interact with histones or may otherwise contribute to the alteration of chromatin structure (*e.g.*, a DNA bend) to inhibit (or enhance) nucleosome assembly.

In the context of conservation of structure and function, inferences gained by the study of centromere function in yeast are expected to be applicable toward the understanding of centromere function in higher organisms. Based on comparisons of the complexity of centromeric DNA, it has been proposed that simple analogies, such as microtubule number per unit centromere-DNA, can be used as an index of evolutionary divergence (13). Additionally, the centromeres of one higher eukaryotic organism have been suggested to have arisen by the fusion of centromeres from different chromosomes, resulting in the increased repetitive nature of the centromeres as well

as an increased chromosome size (12). Furthermore, the observation that large repetitive centromeres can be disrupted into smaller functional elements has led to a model suggesting that these centromeres consist of repeated functional subunits (108). The simple *S. cerevisiae* centromere may represent an evolutionarily basic centromeric unit. Consistent with this view, an analysis of the organization of centromeric chromatin *in vivo* has led to the speculation that the proteins which assemble at the *S. cerevisiae* centromere comprise a fundamentally basic kinetochore (8).

CP1 is a 40 kDal yeast centromere-binding protein which is highly acidic at neutral pH and belongs to the family of basic-helix-loop-helix (b-HLH) sequence specific DNA binding proteins (5, 17). It appears to aid in centromere function by establishing a distinctive chromatin organization in the vicinity of its binding site. The 80 kDal human centromere-binding protein CENP-B is also a highly acidic protein having b-HLH homology (93). Based on the structural similarities between these two proteins, it is tempting to speculate that CENP-B may have a similar function at the human centromere, *i.e.*, binding of CENP-B to human centromeres results in the formation (or preclusion) of a chromatin structure that either facilitates the assembly of other kinetochore proteins or otherwise aids in the maintenance of sister chromatid attachment. Centromere-binding proteins of *Schizosaccharomyces pombe* have not yet been identified, but centromere mutations causing missegregation phenotypes similar to those seen for a CDEI deletion of *S. cerevisiae* centromeres have been described (21). It will be interesting if a similar protein also exists in this organism.

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APPENDIX

**Isolation of the Gene Encoding the *Saccharomyces cerevisiae*
Centromere-Binding Protein CP1**

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ABSTRACT

CP1 is a sequence-specific DNA binding protein of yeast (*Saccharomyces cerevisiae*) which recognizes the highly conserved DNA element I (CDEI) of yeast centromeres. We cloned and sequenced the gene encoding CP1. The gene codes for a protein of molecular weight 39,400. When expressed in *E. coli*, the CP1 gene directed the synthesis of a CDEI-binding protein having the same SDS gel mobility as purified yeast CP1. We have given the CP1 gene the genetic designation *CEP1* (centromere protein 1). *CEP1* was mapped and found to reside on chromosome X, 2.0 centimorgans from *SUP4*. Strains were constructed in which most of *CEP1* was deleted. Such strains lacked detectable CP1 activity and were viable; however, *CEP1* gene disruption resulted in a 35% increase in cell doubling time and a 9-fold increase in the rate of mitotic chromosome loss. An unexpected consequence of CP1 gene disruption was methionine auxotrophy genetically linked to *cep1*. This result and the recent finding that CDEI sites in the *MET25* promoter are required to activate transcription (Thomas, D., Cherest, H., and Y. Surdin-Kerjan, J. Mol. Biol. 9: 3292, 1989) suggest that CP1 is both a kinetochore protein and a transcription factor.

INTRODUCTION

CP1 is a sequence-specific DNA binding protein of yeast (*Saccharomyces cerevisiae*) which binds to both gene promoter regions and centromeres (6). The DNA recognition sequence for CP1 is the octanucleotide RTCACRTG (R=purine) which is found in all yeast centromeres and is named CDEI (centromere DNA element I) (23). The conservation of CDEI strongly implies

a role for CP1 in the function of the centromere or kinetochore; however, CDEI sites are also found at many noncentromeric locations in the yeast genome (5, 6, 16). The existence of noncentromeric CDEI sites together with the fact that CP1 is relatively abundant (greater than 500 molecules per cell) suggests that CP1 may play some general role in chromatin structure or in mediating DNA-protein interactions (5, 6).

Purified CP1 consists of a single polypeptide which appears to have a molecular weight of about 60,000 based on SDS gel analysis (5, 25a). The quaternary structure of the native protein is not known. CP1 is unusually stable; it retains its specific DNA binding activity upon boiling and after elution and renaturation from SDS gels (5, 6). In its stability and DNA binding properties, CP1 is similar to a protein in HeLa extracts which appears to be the adenovirus major late transcription factor (named MLTF or USF) (6, 12, 13, 39). Cai and Davis have purified a CDEI binding protein from yeast which they call CBP-I (9). CBP-I has a molecular weight of 16,000, but shares identical DNA binding properties with CP1. These authors detect no CDEI-binding protein having a higher molecular weight, and they surmise that CBP-I could be derived from CP1, either through a specific post-translational processing event or as a result of nonspecific protein degradation in their nuclear extracts (9).

CDEI is not essential for centromere function, but centromeres lacking an intact CDEI are impaired. Mitotically, chromosomes carrying CDEI mutations are lost at rates 2- to 60-fold higher than the corresponding chromosomes having wild type centromeres (11, 15, 19, 22). Meiotically, chromosomal CDEI mutation or deletion has no detectable effect (19); however, centromere containing plasmids (CEN plasmids) lacking CDEI exhibit random

segregation at meiosis I (15, 19). The effects of CDEI mutations on CP1 binding and chromosome or CEN plasmid loss rates are quantitatively correlated (5, 9), directly implicating CP1 in mitotic centromere function; however, the exact action of CP1 remains obscure. At least one other protein is known to bind to centromeric DNA (21, 35). It binds to another conserved sequence, CDEIII, located approximately 100 bp away from CDEI. CDEIII is essential for proper centromere function (21, 22, 33, 35). Whether or not CP1 and the CDEIII-binding protein interact or influence each other's DNA binding is unknown.

This paper describes the isolation of the gene encoding CP1 and the analysis of yeast strains lacking CP1. We show that the CP1 gene is not essential for growth, but that gene disruption leads to increased rates of chromosome and CEN plasmid loss. Furthermore, CP1 gene disruption has pleiotropic effects on cell metabolism seemingly unrelated to centromere function, and we will present evidence suggesting that CP1 is also a transcription factor.

MATERIALS AND METHODS

Strains and media. Strains used in this study are given in Table 1. JH15 was obtained from Jodi Hirschman of our department; all other strains were constructed by us using standard genetic methods (34). All media were as described by Sherman *et al.* (40) except supplemented minimal medium which was 0.17% yeast nitrogen base (without amino acids), 0.1% ammonium sulfate, 2% glucose, with amino acids (40 $\mu\text{g}/\text{ml}$), adenine (20 $\mu\text{g}/\text{ml}$), and uracil

(20 μ g/ml) added as needed. Yeast transformations were performed by the lithium acetate procedure (25).

Plasmids. Plasmid pDR4-2 contains the 3.6-kilobase pair (kbp) Eco RI fragment of the λ phage CP1-9.1.3 (see below) inserted into the Eco RI site of pGEM4 (Promega Corporation, Madison, WI). Plasmid pRB85a-1 was derived from pDR4-2 by limited Ssp I digestion and religation in the presence of synthetic Xho I linkers. pRB85a-1 lacks the region of DNA between the Ssp I sites located at positions 409 and 1398 in the CP1 DNA sequence (Figure 1). Plasmid pDR8 was obtained by digesting pRB85a-1 with Xho I and Bgl II, filling in the staggered ends using Klenow polymerase, and religating. This results in the removal of an additional 0.6 kbp of CP1 3'-flanking DNA and regenerates both Xho I and Bgl II sites. Plasmid pDR12 was derived from pDR8 by inserting a 1.1-kbp Sal I fragment containing the yeast *URA3* gene (37) into the unique pDR8 Xho I site. Plasmids pDR20 and pDR21 were derived from pUC(TRP/CEN3) and pUC(TRP Δ CDEI), respectively (5), by inserting a 2.2-kbp DNA fragment containing the yeast *LEU2* gene (3) into the Bam HI site of their polylinkers.

The plasmids used for the expression of CP1 in *E. coli* were derived from pTP201 (supplied by Anthony Poteete of our department), which in turn was derived from ptac12 (2) by inserting an Nco I linker at the Pvu II cloning site. pTP201 was cleaved with Nco I, the overhanging ends were filled using Klenow polymerase, and the plasmid was religated in the presence of 8-bp Pvu II linkers. A 1.75-kbp Hind III/Dra I fragment containing the CP1 gene from pDR4-2 was then ligated into the new Pvu II site after Klenow fill-in of the Hind III staggered end. Plasmids pRB89-1 and pRB89-3 resulted from this

ligation. pRB89-1 contains the CP1 open reading frame fused in frame at codon 9 with the ATG originating from the Nco I linker of pTP201. pRB89-3 contains the CP1 segment in the opposite (antisense) orientation. The sequence of the noncoding strand of the fusion segment of pRB89-1 is given below with the CP1 nucleotides and amino acids underlined and the linker sequences shown in lower case:

----- (Tac promoter) ----- AGGAAACAGcccatg gca gAG CTT TCT-----
Met Pro Glu Leu Ser -----

All plasmids containing the Tac promoter were propagated in *E. coli* strain W3110 (*lacI^q*) (7). The predicted molecular weight of the *E. coli* expressed CP1 is 38,800.

Cloning the CP1 gene. Purified CP1 protein (5) was subjected to trypsin digestion, and the partial amino acid sequence of two peptides (NCT14, NCT11) was determined. This analysis was performed at the Harvard Microchemistry Facility using the method described by Aebersold *et al.* (1). The peptide sequences were used to design two nondegenerate oligonucleotide probes, shown below:

NCT14

AspGlnGlyLeuLeuSerGlnGluSerAsnAspGlyAsnIleAspSerAlaLeuLeuSer 5'-
GATCAAGGTTTGTGTCTCAAGAATCTAACGATGGTAACATTGATTCTG
C-3'

NCT11 AlalleThrProSerAsnGluGlyValLysProAsnThr[Gly/Ser]Leu

5'-GCTATTACTCCATCTAACGAAGGTGTTAAGCCAAACAC-3'

In all but one case (AAG for Lys), we chose codons predicted from yeast codon usage frequencies supplied with the DNASTAR computer software package (DNASTAR, Inc., Madison, WI). The NCT14 and NCT11 oligonucleotides were synthesized, labeled with [α - 32 P]ATP using T4 polynucleotide kinase, and used to screen a commercially available (Clontech, Palo Alto, CA) yeast genornic λ gt11 library (*Saccharomyces cerevisiae* strain X2180, ATCC #26109) by hybridization (24). Positive signals were obtained at a frequency of about 1 per 3,500 recombinant plaques. Two phage which hybridized to both probes were purified and found to contain overlapping yeast DNA inserts. One of them, λ CP1-9.1.3, contained a 3.6-kbp Eco RI insert which was subcloned into both plasmid and M13 vectors.

The DNA sequence of the CP1 gene was determined by the dideoxy chain termination method using modified T7 DNA polymerase (41). The CP1 coding region was located immediately by using the NCT14 and NCT11 oligonucleotides as sequencing primers. Restriction sites discovered in these initial sequence runs were then used to map the location of the gene within the 3.6-kbp Eco RI fragment and to produce additional subclones. The CP1 DNA sequence presented in Figure 1 was determined completely for both strands. A homology search of the National Biomedical Resource Facility protein database (release 21) was performed against the deduced CP1 amino acid sequence. The search employed the Lipman-Pearson search algorithm (3 1) and was carried out by the PROSCAN computer program of DNASTAR.

Southwestern blots. *E. coli* containing the Tac-CP1 expression plasmids pRB89-1 (sense) and pRB89-3 (antisense) were grown and induced with isopropyl-p-D-thiogalactoside (IPTG) as described by Amann *et al.* (2). After induction for 90 min, the cells from 1-2 ml of culture were sedimented by brief centrifugation and resuspended in 75-150 μ l of SDS sample buffer [63 mM Tris-HCl (pH 6.8), 2% SDS, 5% BME, 10% glycerol, 0.002% BPB]. The ratio of cells to buffer was kept constant in order to obtain equivalent protein concentrations among samples; 0.6 OD 600 "units" of cells were resuspended per 75 μ l of sample buffer. The samples were incubated in a boiling water bath for 5 min, and then 5 μ l (for Coomassie staining) or 10 μ l (for blotting) of each were immediately loaded onto a 10% or 12% polyacrylamide (acrylamide:bis, 37.5:1) SDS gel prepared and run using the discontinuous buffer system of Laemmli (29). After electrophoresis, proteins were transferred to nitrocellulose membranes (Biorad) following the electroblotting procedure of Masudaira (32).

After protein transfer, the membranes were blocked for 1 h at room temperature in 10 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1 mM DTT, 1% nonfat dry milk and then incubated for 30 min in binding buffer [10 mM HEPES (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT containing ³²P-labeled CDEI DNA (7 ng/ml, 5 x 10⁴ cpm/ng) and 5 μ g/ml salmon sperm DNA. Unbound probe was removed by two 7 min washes in binding buffer, and the membrane was blotted dry and used to expose X-ray film. In order to estimate protein molecular weights, prestained molecular weight standards (Bethesda Research Laboratories) were co-electrophoresed on the SDS gels and transferred to the membranes along

with the sample proteins. Before autoradiography, the positions of the standards were marked with Glo-juice phosphorescent ink (International Biotechnologies, Inc.) allowing their direct identification on the autoradiograms.

The CDEI probe was a multimer (50-300 bp) of the synthetic double stranded oligonucleotide shown below (CDEI site underlined):

5'-GATCCAAATAAGTCACATGATGATA-3'

3'-GTT TAT TCAGTGTACTACTATCTAG-5'

The oligonucleotide was phosphorylated, self-ligated, and labeled with [α - 32 P]dCTP (800 Ci/nimol) by nick translation (43). CDEI competitor DNA was prepared by the same method, but it was of high molecular weight (>500 bp) and unlabeled.

Detection of CP1 in yeast extracts. Crude yeast extracts were prepared and gel shift DNA binding assays were performed as described previously (5). The CDEI probe consisted of a hexamer of the synthetic CDEI oligonucleotide shown above which was cloned into the Bam HI site of pUC18, excised as a 205-bp Eco RI/Hind III fragment, and end-labeled with [α - 32 P]dATP (800 Ci/mmol) using Klenow polymerase. The tDNA probe was a 480-bp Bgl II fragment containing the yeast tRNA^{Ser} gene (4) end-labeled with [α - 32 P]dATP (800 Ci/mmol) using Klenow polymerase (in the presence of unlabeled dGTP). Each binding reaction contained 10^4 cpm of probe (2-10 ng) and 6 μ g of unlabeled poly[d(IC)]. Where indicated, binding reactions received 5 μ g of un-

labeled poly[d(IC)] and 1.0 μ of the self-ligated CDEI competitor DNA described above.

CP1 gene disruption. We have designated the genetic locus of the CP1 gene as *CEP1* for centromere protein 1. *CEP1* gene disruption was performed using the plasmid pDR12 in which most of the CP1 coding region (codons 53-351) and 0.6-kbp of 3'-flanking DNA is replaced by the yeast *URA3* gene (see above). pDR12 was cleaved with Eco RI to excise the disrupted CP1 gene (*cepl::URA3*) from the vector and then used to transform the diploid strain JHI5. Homologous recombination stimulated by the free ends of the transforming DNA results in the replacement of the wild type CP1 gene with the *cepl::URA3* disruption allele (38). Ura⁺ transformants were analyzed by Southern blotting, and most were found to contain one copy of the disrupted CP1 gene and one copy of the wild type gene. One such transformant, R11, was sporulated and the tetrads dissected. The *URA3* marker segregated 2⁺:2⁻, and Southern blotting showed that the ura⁺ spores gave rise to restriction fragments diagnostic of the gene disruption and ura⁻ spores to fragments diagnostic of the wild type gene, *i.e.* the physical and genetic markers cosegregated.

Chromosome and plasmid loss assays. Chromosome III loss rates in the diploid strains R16, R17, and R18 were determined by quantitative mating (36). The haploid tester strain employed was 4795-408 (MATa). Colonies of the diploids were grown on plates lacking leucine to require maintenance of the MATa *LEU2* chromosome. For each determination, a single colony was re-suspended in water, sonicated briefly, and used to inoculate a 100-ml rich broth (YEPD) culture. An aliquot of the cell suspension was also diluted and

plated on YEPD plates to determine the starting cell number. The YEPD liquid cultures were grown at 30° to mid log phase (16-18 h) and the cell density determined. In duplicate, 2×10^6 of the diploid cells were mixed with 2×10^6 of the haploid tester cells, also from a mid log phase culture, and filtered onto a 25mm Millipore filter. Filters were incubated cell side up on YEPD plates at 30° for 4.5 h to allow mating. The mating mixtures were then resuspended from the filters in 5.0 ml of water, diluted, and spread on plates lacking histidine and lysine to select for growth of zygotes. In each experiment, haploid strain R11-4D (MAT α) was mated with 4795-408 under the same conditions in order to establish the mating efficiency. After 48 hours, colonies were counted and then replicated onto plates lacking leucine to test for the presence of the *LEU2* marker originally present on the MATa chromosome. Leu⁻ colonies were assumed to have arisen from cells which had become competent to mate due to loss of the MATa*LEU2* chromosome occurring during the nonselective growth of the culture. The frequency of leu⁻ zygotes (corrected for mating efficiency) is therefore an indirect measure of chromosome loss frequency. Chromosome loss rates were calculated from the following formula (17):

$$\text{loss rate} = (0.43)(\text{loss frequency})/(\log N - \log N_0)$$

N and N₀ are the number of cells in the population at the end and start of nonselective (YPD) growth, respectively. Control experiments showed that the *cepl::URA3* allele does not affect the mating efficiency of haploid strains of either mating type (data not shown). We do not know if chromosome III monosomy in our diploid strains leads to any growth defect. If it

does, our results would be skewed. Mating competent cells (loss events) would be underrepresented in the total cell population, leading to an underestimation of the loss rate.

Plasmid loss determinations were carried out essentially as described (5) except the plasmid selectable marker was *LEU2*. Individual transformant colonies were grown selectively, either in liquid or on plates, to maintain the CEN plasmid. After selective growth, cell suspensions were used to inoculate liquid YEPD cultures and at the same time diluted and spread on YEPD plates to obtain single colonies (t_0). After 18-20 h of growth at 30°, the liquid YEPD cultures were diluted and plated for single colonies on YEPD plates (t_n). After colonies had formed (2 days), the t_0 and t_n plates were replicated onto supplemented minimal plates with or without leucine, and after 24 h the percentage of *leu*⁺ colonies was determined. The number of cell doublings was calculated by determining the total cell number at the start and end of nonselective (YEPD) growth. Plasmid loss rates were then calculated from the following equation (27):

$$L_n/L_0 = (1-R)^n$$

L_n and L_0 are the percentage of *leu*⁺ colonies after nonselective and selective growth, respectively, n is the number of doublings during nonselective growth, and R is the plasmid loss rate (loss events/cell/generation).

RESULTS

Isolation of the gene encoding CP1. Purified CP1 was cleaved with trypsin, and the partial amino acid sequences of two tryptic peptides, NCT14 and NCT11, were determined. The sequences were used to design two oligonucleotide probes, a 50-mer from NCT14, and a 38-mer from NCT11, which were synthesized, labeled, and used to screen a λ gt11 yeast genomic library by hybridization (details are given in Materials and Methods). Two different phage were obtained which hybridized to both probes, and the 3.6 kbp Eco RI insert from one of them was subcloned for DNA sequence analysis. The complete DNA sequence of the CP1 gene is given in Figure 1 along with the deduced CP1 amino acid sequence. The gene codes for a protein of molecular weight 39,400. A homology search of the National Biomedical Resource Facility protein sequence database against the predicted CP1 amino acid sequence revealed no striking similarity to any protein in the database.

The molecular weight of CP1 determined by SDS gel electrophoresis is 58,300 (5), significantly larger than that of the polypeptide encoded by the putative CP1 gene. Of the several possible explanations for this discrepancy, we thought it to be most likely that the CP1 protein contains post-translational modifications which affect its mobility in SDS gels. To test this and at the same time prove that the open reading frame we identified actually codes for CP1, we expressed the gene in *E. coli*. A hybrid transcription unit was constructed by fusing the CP1 open reading frame at the naturally occurring Hind III site at CP1 codon 9 to the *E. coli* Tac promoter (2). An initiator ATG was provided by the Tac promoter fragment, and the molecular weight of the expected translation product is 38,800.

Proteins produced by *E. coli* strains carrying Tac-CP1 expression constructs were analyzed by "southwestern blotting". Proteins were separated on SDS gels and transferred to nitrocellulose membranes which were then incubated with a ^{32}P -labeled synthetic DNA containing CDEI, *i.e.* the recognition site for CP1. Fig. 2A shows the results for bacteria grown in the presence or absence of IPTG which is required to induce transcription of the Tac promoter. Upon induction, a protein band is detected which binds the CDEI probe (Fig. 2A, lane 1). The binding activity is not present in protein extracts prepared from the same *E. coli* strain grown in the absence of IPTG (Fig. 2A, lane 2), nor is it observed in induced strains carrying a Tac-CP1 construct in which the CP1 segment was inserted in the opposite (antisense) orientation (Fig. 2B, lane 2). Binding is competed by excess unlabeled CDEI-containing DNA (Fig. 2B, lane 3), but not poly[d(IC)] or salmon sperm DNA.

Based on its mobility relative to prestained electrophoresis standards, the *E. coli*-produced CDEI-binding protein has a molecular mass of 55-60 kDa. Close examination of Coomassie blue stained gels reveals that a new protein band is present in this region of the gel (Fig. 2B and 2C, lanes 5); its apparent molecular-weight is 58,300 based on the Coomassie blue-stained standards. While not prominent, the appearance of this protein band correlates exactly with the synthesis of the binding activity, *i.e.* it is present only after IPTG induction and in strains carrying the sense Tac-CP1 construct. The *E. coli*-synthesized protein, then, has the same mobility on SDS gels as purified yeast CP1. We conclude that the gene we have isolated does indeed code for the CDEI binding protein CP1. Since we are unaware of any posttranslational modifications common to yeast and *E. coli* which would explain such a large discrepancy be-

tween the predicted molecular weight and that which is observed, we also conclude that the CP1 polypeptide migrates aberrantly in SDS gels.

Construction of a yeast strain with a disrupted CP1 gene. As a first step towards elucidating the function of CP1 in the cell, we constructed yeast strains in which the gene encoding CP1 was disrupted. Starting with a plasmid containing the cloned CP1 gene, most of the coding region (codons 53-351) along with 3'-flanking DNA was excised and replaced with a DNA fragment carrying the yeast *URA3* gene. The disrupted gene along with its remaining flanking regions was then excised from the plasmid and used to transform a diploid homozygous *ura3* strain. *Ura*⁺ transformants were obtained in which the disrupted gene construct had replaced the resident CP1 gene on one of the two chromosomes. One such strain, R11, was analyzed by Southern blotting and found to have the expected CP1 gene configuration, i.e. restriction digests yielded one fragment derived from the parental gene and a second fragment derived from the disrupted gene (data not shown). When R11 was sporulated and the asci dissected, most asci gave rise to three or four viable spores, indicating that the CP1 gene is not essential. The *URA3* marker, physically linked to the disrupted CP1 gene, segregated 2⁺:2⁻. We designated the genetic locus of the CP1 gene *CEP1* for centromere protein 1.

The *CEP1* locus was mapped, first by chromosome blotting and subsequently by genetic linkage analysis. Hybridizing a Southern blot of electrophoretically separated yeast chromosomes with a *CEP1* probe allowed us to assign *CEP1* to chromosome X (data not shown). When genetic linkage to other chromosome X markers was tested, we found that *CEP1* mapped very

close to *SUP4*. Table 2 gives the results of tetrad analysis. The genetic distance between *CEP1* and *SUP4* is 2.0 centimorgans.

CDEI binding activity in *CEP1*⁺ and *cepl::URA3* strains. Extracts were prepared from haploid segregants of R11 and assayed for CDEI binding activity by gel shift assay. When a *CEP1*⁺ extract (R11-4C) is incubated with a CDEI probe, a predominant CP1-DNA complex is formed as well as lesser amounts of two or more complexes of lower mobility (lane 3). The lower mobility complexes are probably due to oligomers of CP1. All of these complexes are also formed by purified CP1 (lane 2) and are greater than 95% competed by excess unlabeled CDEI-containing DNA (lanes 5 and 6; quantitation by densitometry). [The complexes observed in lane 2 migrating beneath the major CP1-DNA complex are due to partial degradation of CP1 which occurs during its purification; they are competed by excess CDEI DNA.] An extract prepared from a *cepl::URA3* strain (R11-4B) fails to form any of the CP1-DNA complexes (lane 4), although a faint, smeared signal can be detected in the region of the gel at the position of the low mobility complexes. We do not feel that this weak signal is due to residual CP1, because in dozens of gel shift assays with partially pure or pure CP1, we have never observed low mobility CP1 complexes in the absence of a major CP1-DNA band. A distinct band which runs below the main CP1-DNA complex is observed with both the R11-4B and R11-4C extracts, but this band is due to a nonspecific binding activity since it is not competed at all by CDEI DNA.

To insure that our failure to detect CP1 activity in the R11-4B extract was not simply due to improper handling of the extract or to some general effect of the *CEP1* disruption on DNA binding proteins (e.g. increased levels of

protease), we simultaneously assayed for another sequence specific DNA binding protein, transcription factor τ . Factor τ is an RNA polymerase III transcription factor which binds to the internal promoter of tRNA genes (10). Factor τ binding activity is readily detected in both R11-4C and R11-4B crude extracts using a tDNA^{Sex} probe (lanes 15 and 16). When both probes are added together, both CP1 and τ complexes are observed with the R11-4C extract (lane 9), but only τ complexes are observed with the R11-4B extract (lane 10). Only the CP1 complexes are competed by CDEI DNA (lanes 11-13). We conclude that the *cep1::URA3* disruption results in the loss of functional CP1 protein from the cell.

Phenotypic effects of *CEP1* gene disruption. Haploid strains carrying the *cep1::URA3* gene disruption are relatively healthy; however, we noticed immediately that *cep1::URA3* disruptants grow slower than isogenic *CEP1*⁺ strains. The doubling times for several strains, both haploid and diploid, growing in rich medium at 30° are given in Table 3. The *cep1::URA3* gene disruption leads to a 35% lengthening in the population doubling time, from about 95 to about 130 minutes. The growth defect is recessive; *cep1::URA3*/+ heterozygous diploids grow at the same rate as *CEP1*⁺ haploids or homozygous *CEP1*⁺ diploids.

Since CP1 binding seems to be required for optimal mitotic function of the centromere (5), a yeast strain lacking CP1 should exhibit increased rates of chromosome loss. Chromosome III loss rates in *cep1::URA3* strains were determined using a quantitative mating assay (14). The assay is based on the fact that a *MATa*/*MAT α* diploid cell, a nonmater, can become competent to mate if it loses one of its chromosome III homologs. [The mating type locus, *MAT*,

is located on the right arm of chromosome III.] Chromosome III loss results in a cell becoming phenotypically a or α , depending on which homolog is lost, and able to mate with a haploid cell of the opposite mating type. An a/α diploid can also become competent to mate through mitotic recombination or gene conversion at the MAT locus; however, these events can be distinguished from chromosome loss experimentally by scoring for the concomitant loss of a genetic marker on the opposite arm of chromosome III.

Table 4 gives the results of two separate experiments in which we measured rates of chromosome III loss from the diploid strains R16 (*cep1::URA3/cep1::URA3*), R17 (*CEP1⁺/cep1::URA3*), and R18 (*CEP1⁺/CEP1⁺*). The rate of chromosome III loss from the wild type diploid (R18) was $1-2 \times 10^{-5}$ events/cell/division. This value agrees with previous measurements of chromosome III loss (14, 18). When homozygous, the *cep1::URA3* disruption caused a 9-fold increase in the rate of chromosome III loss. As with the slow growth phenotype, the gene disruption was recessive in its effect on chromosome loss. In fact, the rate of loss in the *cep1::URA3/+* heterozygote (R17) seemed to be even lower than that of the wild type diploid (R18). This 2-fold difference was observed consistently, but at present we are unable to provide any explanation for it. No significant differences were observed among these three strains in the combined rates of mitotic recombination and gene conversion (data not shown).

Rates of minichromosome (i.e. CEN plasmid) loss were also determined for wild type and *CEP1* disruption strains. In this case, it was possible to directly compare the *cis* effect of a CDEI mutation with the *trans* effect of disrupting *CEP1*. If the only involvement of CP1 in mitotic chromosome segregation is

exerted through its binding to CDEI at the centromere, then the effect of deleting CDEI from the centromere in a normal cell should be quantitatively similar to the effect on a wild type centromere when CP1 is absent from the cell. Loss rates were measured for two CEN plasmids, pDR20 and pDR21, in *CEP1*⁺ and *cepl::URA3* haploid strains. The plasmids are identical except that pDR20 contains a wild type CEN3 segment whereas the CEN segment in pDR21 lacks CDEI owing to a 14 bp deletion. The plasmids also contain the selectable markers *TRP1* and *LEU2* and the replicator sequence (origin) *ARS1*.

Previous experiments (5) had shown that the CDEI deletion in pDR21 leads to an increased rate of plasmid loss, but actual loss rates were not determined. Table 5 shows that pDR20 was lost from *CEP1*⁺ cells at a rate of 1.6%/division; the loss rate of pDR21 was increased 2.5-fold to 3.9%/division. The rate of loss of the wild type CEN plasmid from the *cepl::URA3* strain was 3.2%/division, not significantly (or statistically) different from the rate of loss of the CDEI deleted plasmid from the wild type cell. Furthermore, the effects of the mutations were not additive; the mutant CEN plasmid was lost from the *cepl::URA3* strain at a rate of 3.6%/division. We conclude that the involvement of CDEI in mitotic centromere function is mediated through the *CEP1* gene product, CP1.

Methionine auxotrophy. Several researchers have noted that CDEI sites exist in many noncentromeric locations throughout the yeast genome. Several genes contain CDEI sites in their 5'-flanking regions where they could conceivably serve as transcriptional activation sequences (UAS's), among them, *MET2*, *MET25*, *ADE3*, *GPA2*, and *SRA3*(from a computer search of GenBank, release 58), and three nuclear genes encoding mitochondrial pro-

teins (16). To determine if CP1 gene disruption caused any metabolic deficiency, we surveyed the growth requirements of our *cep1::URA3* strains. Under all conditions tested, *cep1::URA3* strains grew slower than their isogenic *CEP1*⁺ counterparts; however, the growth disadvantage was accentuated at 22° and on medium containing lactate as the sole carbon source. *CEP1*⁺ and *cep1*⁻ strains grew at about the same relative rates on rich medium (YEPD) at 30° and 34° and on plates containing glycerol as the carbon source (YEPG). Surprisingly, we found that *cep1::URA3* strains required exogenously added methionine for growth. The methionine auxotrophy was genetically linked to the *cep1::URA3* disruption (Table 2). In 53 tetrads dissected from a *cep1::URA3*/+ diploid, no *ura*⁺*met*⁺ or *ura*⁻*met*⁻ spores were obtained, meaning that the lesion which gives rise to the methionine auxotrophy was not genetically separable from the *cep1::URA3* gene disruption. [The unlinked *tyr1* marker (chromosome II) showed the 1:1:4 segregation pattern expected.] Formally, the genetic distance between *cep1::URA3* and *met*⁻ is less than 0.9 centimorgan (one tetatype in 54 meioses). We interpret this result to mean that the *cep1::URA3* gene disruption in fact causes the methionine auxotrophy. None of the 21 genetically defined *met* genes has been mapped to this region of chromosome X, although several have not yet been mapped (26).

DISCUSSION

We have cloned the gene encoding the yeast centromere binding protein CP1. The molecular weight of CP1 predicted from its DNA sequence is 39,400, considerably less than the apparent molecular weight of the purified protein which is 58,000 based on SDS gel analysis. However, when expressed

in *E. coli*, the cloned CP1 gene directs the synthesis of a protein whose mobility in SDS gels is indistinguishable from that of native yeast CP1 and which specifically binds to DNA containing CDEI, the DNA recognition sequence of CP1. Furthermore, the deduced CP1 protein sequence contains exact matches to the partial amino acid sequences that were determined for two CP1 tryptic peptides. These results along with the genetic evidence discussed below leave little doubt that the isolated gene does indeed encode CP1. Evidently, CP1 migrates aberrantly in SDS gels. The predicted CP1 protein sequence contains 20% acidic residues (glutamate, aspartate) which results in the protein having a predicted net charge of -20 at pH 7. Whether or not it is this strongly acidic character or perhaps an unusual secondary structure which leads to the anomalous SDS gel mobility is unknown. A similar situation exists with the yeast protein ARS binding factor 1 (ABF1)(15a). The *ABF1* gene encodes a polypeptide of molecular weight 81,759, yet purified ABF1 migrates on SDS gels with an apparent molecular weight of 135,000. Like CP1, ABF1 has a high aspartic acid content (11.4%) and high net negative charge at neutral pH (-6.0). The yeast transcription factors HSTF (heat shock transcription factor) and RAP1 also migrate slowly in SDS gels (references in 15a).

Cloning the CP1 gene, which we designate *CEP1*, enabled us to obtain yeast strains in which this gene is disrupted. Haploid cells containing a disrupted *CEP1* gene are viable, indicating that *CEP1* is nonessential. However, *cep1⁻* strains grow more slowly than their isogenic *CEP1* counterparts, exhibit increased rates of mitotic chromosome and CEN plasmid loss, and are methionine auxotrophs. To construct the *cep1::URA3* disruption allele (strain R11-4B), 900 bp of coding region and about 700 bp of 3'-flanking DNA were

deleted. A possible explanation for the pleiotropic effects of this mutation would be that the deletion of downstream sequences has disrupted a neighboring gene. Three lines of evidence (data not shown) argue against this possibility. First, strains containing an insertion of the yeast *LEU2* gene 700 bp downstream of *CEP1* (the *Bgl* II site) grow normally and are methionine prototrophs. Second, a haploid strain containing a *cep1::URA3* disruption which removes only coding region and 90 bp of downstream sequences (to the *Ssp*I site at position 1398, Figure 1) has a phenotype indistinguishable from that of R11-4B--i.e. slow growth, increased CEN plasmid loss, methionine auxotrophy. Third, the methionine auxotrophy linked to the R11-4B gene disruption is complemented by a CEN plasmid containing the *CEP1* gene having only 110 bp of 3'-flanking DNA.

The observed increase in culture doubling time of *cep1*- strains is about 35%, while chromosome loss rate is increased 9-fold. Mathematical modeling (not shown) reveals that in order to observe a 35% increase in population doubling time, chromosome loss (or any event leading to cell inviability) would have to occur in about 10 out of 25 mitoses or 40%/cell/division. The increased rate of chromosome loss, from about 10^{-5} loss events/cell/division in a wild type strain to 10^{-4} loss events/cell/division in a *cep1*- strain, is not nearly sufficient to account for the decreased rate of culture growth. Also, we do not observe an accumulation of inviable cells in cultures of *cep1* strains. Although other explanations are possible, we suggest that *cep1*- cells actually have longer cell cycle times, perhaps implying that *CP1*'s role in cell metabolism is not confined to mitosis.

Gel shift DNA binding assays performed with extracts from haploid *cep1::URA3* cells revealed no detectable CP1. This was not surprising, since the results of genomic Southern blots had indicated that the *CEP1* gene is unique. But is CP1 the only CDEI-binding protein in the cell? It is not totally inconceivable, for instance, that CP1 carries out a function related to noncentromere CDEI sites while an as yet unrecognized, centromere-specific CDEI binding protein is responsible for CDEI-related centromere function(s). Our DNA binding assay results argue against such a scenario. No clear, CDEI-specific binding activity is detected at all in *cep1⁻* cells. A weak, indistinct gel shift signal is observed, but it could comprise at most 6% (quantitation not shown) of the CDEI-binding activity present in *CEP1⁺* cells. We favor the view that CP1 is the only CDEI-binding protein in yeast; however, our data do not rule out the existence of others.

Point mutations or deletion of CDEI from the centromeres of plasmid minichromosomes result in small but significant decreases in their mitotic stability (5, 22, 35a). Our results here show that the increase in plasmid loss rate due to CDEI deletion is about 2.5-fold. Hegemann *et al.* tested the effect of several CDEI point mutations on the mitotic stability of 150-kbp chromosome fragments and found 2- to 10-fold elevated loss rates depending on the mutation (22). The effect of deleting CDEI from CEN3 is a 20- to 60-fold increase in the rate of mitotic chromosome loss (15, 19). We find that a cell lacking CP1 loses chromosome III at a rate 9-fold higher than that of a wild type cell. This observed increase in mitotic loss rate is somewhat less than might be expected from the results of the CDEI deletion analyses just described but is still consis-

tent with our assertion that the mitotic function of CDEI is mediated through CP1 (5).

The *trans* effect of removing CP1 from the cell and the *cis* effect of deleting CDEI from the centromere were directly compared by measuring the loss rates of CEN plasmids from wild type and *cep1::URA3* strains. The rate of loss of a wild type CEN plasmid from a *cep1*⁻ cell is not significantly different than the rate of loss of a CDEI-deleted CEN plasmid from a *CEP1*⁺ cell. Furthermore, the *cis* and *trans* effects are not additive in that the rate of loss of the CDEI-deleted plasmid in the *cep1*⁻ strain is not further increased. These results indicate that the impaired function of CDEI-mutated centromeres can be attributed to the lack of CP1 interaction. It follows then that CP1 is directly involved in centromere function, and that no other CDEI-binding protein exists which can fulfill this role.

CDEI sites are found at several noncentromere locations in the yeast genome, in some instances in gene promoter regions (5, 6, 16). In fact, in their original paper, Bram and Komberg speculated that in some contexts CP1 might function as transcription factor (6). Interestingly, CP1 has a seemingly identical DNA binding specificity as the human transcription factor MLTF. However, partially purified CP1 cannot substitute for MLTF in a reconstituted mammalian *in vitro* transcription system (13). Recently, Thomas *et al.* discovered that CDEI sites upstream of the yeast *MET25* gene (coding for O-acetylhomoserine sulfhydrylase) are required for transcriptional activation (42). When the CDEI sequences are deleted, enzyme activity is decreased by over 90% and a corresponding decrease is observed in the steady state levels of *MET25* mRNA (42). Strains with *met25* mutations require exogenously

added methionine for growth. It seems highly likely that the methionine auxotrophy of our *cep^{cep1}* strains is caused at least in part by an inability to transcribe *MET25*. CDEI sites are also found in the promoter regions of *MET2*, *MET3*, *SAM1*, and *SAM2*, genes which are co-regulated with *MET25* (42). This implicates CP1 in the transcriptional control of several genes involved in methionine metabolism, and it is possible that it is the disruption of these inter-related pathways which leads to the growth defect of *cep1⁻* strains even when growing on rich medium.

As yet we have no direct evidence that CP1 functions as a transcription factor. We are in the process of analyzing *MET25* transcription in our *cep1::URA3* strains, and the results will tell whether or not CP1 is required for *MET25* expression. If CP1 is a transcription factor, we would like to know how it performs its dual role. Does it carry out exactly the same function at the centromere as it does at a gene promoter? Does it interact with the same or different proteins at its various sites of action? The inferred multifunctional nature of CP1 is reminiscent of another yeast DNA binding protein, general regulatory factor I (GRFI). GRFI (also known as RAP1) binds to a sequence element found in gene promoters (*MAT α 1/ α 2*, *TEF2*), in the silencer regions of the *HML* and *HMR* mating type loci, and in yeast telomeres, thereby suggesting roles for GRFI in transcriptional activation, transcriptional repression, and chromosome structure (8). Both CP1 and GRFI are relatively abundant (5, 6, 8). Buchman *et al.* have suggested that GRFI and CP1 are members of a class of chromatin structural components which act in conjunction with other factors to build the chromatin structures necessary for both gene expression and chromosome maintenance (8). Our results with CP1 are not inconsistent with

that view. More specifically, we are intrigued by the possibility that CP1 and/or GRFI might bind to their cognate sites in DNA and then direct nuclear matrix attachment or determine intranuclear location. Cloning *CEP1* has allowed us to begin the genetic analysis of CP1 function. Hopefully, the detailed study of *cep1* mutants will enable us to further elucidate the role(s) of CP1 in cellular metabolism.

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TABLE 1. Strains

Strains ^a	Genotype
381G	<i>a cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-a</i>
R11-4C	381G <i>a leu2 ura3 bar1-1 TYR1</i>
R11-4D	381G α <i>leu2 ura3 bar1-1 TYR1</i>
R15-3A	381G α <i>leu2 ura3 bar1-1(?) TYR1</i>
R11-4B	381G α <i>leu2 ura3 bar1-1 TYR1 cep1::URA3</i>
R11-15C	381G <i>a leu2 ura3 bar1-1 TYR1 cep1::URA3</i>
R15-3B	381G <i>a leu2 ura3 cep1::URA3</i>
JH15	<u>381G <i>a leu2 ura3 bar1-1 TYR1</i></u>
	381G α <i>leu2 ura3 bar1-1 TYR1</i>
R11	<u>381G <i>a leu2 ura3 bar1-1 TYR1 cep1::URA3</i></u>
	381G α <i>leu2 ura3 bar1-1 TYR1</i> <u>CEP1</u>
R16	<u>381G <i>a LEU2 ura3 BAR1 tyr1 cep1::URA3</i></u>
	381G α <i>leu2 ura3 bar1-1 TYR1 cep1::URA3</i>
R17	<u>381G <i>a LEU2 ura3 BAR1 tyr1 cep1::URA3</i></u>
	381G α <i>leu2 ura3 bar1-1 TYR1</i> <u>CEP1</u>
R18	<u>381G <i>a LEU2 ura3 BAR1 tyr1</i></u> <u>CEP1</u>
	381G α <i>leu2 ura3 bar1-1 TYR1</i> <u>CEP1</u>
4795-408 ^b	<i>a leu2 ade2 ade3 his7 trp1 can1 sap3</i>
J15-13C ^c	α <i>ade2-1 lys2-1 trp5-2 leu1-12 can1-100 met4-1 ura1-1 SUP4-o</i>
D2	R15-3B crossed with J15-13C

^aAll except the last three strains are congenic to strain 381G(20)

^bCongenic to A364A, obtained from L. Hartwell, University of Washington.

^cKurjan and Hall (28); SUP4-o was scored by its ability to suppress *ade2-1* (ochre).

TABLE 2 Tetrad analysis

Diploid	Interval	PD ^a	NPD	T	Map distance ^b (centimorgans)
D2	<i>cep1::URA3-SUP4</i>	72	0	3	2.0
R17	<i>cep1::URA3-met⁻</i>	53	0	0	0.0
	<i>cep1::URA3-tyr1</i>	13	7	32	NL ^c

^aPD, parental ditype; NPD, nonparental ditype; T, tetratype.

^bMap distance = $[T + 6(NPD)] / 2(PD + NPD + T) \times 100$ (34).

^cNo linkage.

TABLE 3. Doubling Times of *CEP1*-Disrupted Strains^a

Strain	<i>CEP1</i> Genotype	Doubling Time ^b (min.)
Haploids		
R11-4C	<i>CEP1</i> ⁺	98 ± 4 (2)
R15-3A	<i>CEP1</i> ⁺	95
R11-4B	<i>cep1::URA3</i>	128 ± 4 (2)
R15-3C	<i>cep1::URA3</i>	133
Diploids		
R18	<i>CEP1</i> ⁺ / <i>CEP1</i> ⁺	98
R17	<i>CEP1</i> ⁺ / <i>cep1::URA3</i>	94
R16	<i>cep1::URA3/cep1::URA3</i>	127

^aCells from late log cultures were diluted into fresh YEPD medium and grown at 30°. Aliquots were removed at various times, and, after brief sonication to separate cells, the cell number was determined using a Coulter counter.

^bDoubling times were determined from semilog plots, fitting the curves by the method of least squares. Where more than one determination was made (number in parentheses), the result is reported as the mean ± the total deviation.

TABLE 4. Chromosome III Loss Rates in *CEP1*-Disrupted Strains

Strain	CEP1 Genotype	Loss Rate ^a	
		Exp.1	Exp.2
Events/cell/generation			
R18	CEP1 ⁺ /CEP1 ⁺	2.2x10 ⁻⁵	1.1x10 ⁻⁵
R17	CEP1 ⁺ /cep1::URA3	1.4x10 ⁻⁵	0.4x10 ⁻⁵
R16	cep1::URA3/cep1::URA3	1.9x10 ⁻⁴	1.0x10 ⁻⁴

^aLoss Rates were determined as described in Materials and Methods. The strain stocks used in experiments 1 and 2 were totally independent.

TABLE 5. CEN plasmid Loss Rates

Strain	Plasmid	%loss/cell/generation ^a
R11-4C (<i>CEP1</i> ⁺)	pDR20 (CEN)	1.6 ± 0.2 (9)
R11-4C (<i>CEP1</i> ⁺)	pDR21 (Δ CDEI)	3.9 ± 0.8 (8)
R11-4B (<i>cep1::URA3</i>)	pDR20 (CEN)	3.2 ± 0.3 (9)
R11-4B (<i>cep1::URA3</i>)	pDR21 (Δ CDEI)	3.6 ± 0.3 (8)

^aThe data from three separate experiments were pooled; the number of independent determinations for each strain/plasmid combination is given in parentheses. The results are reported as mean ± SEM. By the F test (30), the only statistically significant difference among the means ($P > 0.95$) was the loss rate of plasmid pDR20 in strain R11-4C which was different from each of the other three.

	GGATCCTTCGGGACCACCA ⁺ GTCTCACCCACCAAGGTCA ⁺ AAAAAATAAAATCGGACGAG ⁺ AAAGTATTGGGCAAAACA ⁺ CAATTATCCACGTGCTTATCA	100
	CGTTTTTTTATTTAATGTAAGATGTAACCATTATACCT ⁺ TAAAGATAAACACTTTGTTA ⁺ ATTATTCTGTTTAGTTGGCGGTCATTTTCATTTCTGCTACA	200
	AGTACCAACATCAAGTGC ⁺ TAAAAATAATACGGTTTTCTACACTTTTATTAACGATGA ⁺ ACTCTCTGGCAAATAATAA ⁺ TAGCTTTCTACTGAGGATGAG	300
	M N S L A N N N K L S T E D E	
	GAAATCCATTTCGGCAAGAA ⁺ AAAGAGGCTATAACGAGGAG ⁺ CAGAACTACAGCGAGGCCAG ⁺ AAAAAGCAAAGAGATCAAG ⁺ GCTTGTGTGCACAAGAGAGCA	400
16	E I H S A R K R G Y N E E Q N Y S E A R K K Q R D Q G L L S Q E S N	
	NCT14	
	^{Ssp I} ATGACGGAAATAT ⁺ TGACTCTGCTCTGTTGAGCGAAGGGG ⁺ CTACGCTAAAAGGACTCAA ⁺ AGTCAGTATGAAAGCGGACTGACGCTTAACAAAGATGAGAA	500
50	D G N I D S A L L S E G A T L K G T Q S Q Y E S G L T S N K D E K	
	AGGAAGTGATGACGAAGATG ⁺ CGTCAGTGGCTGAGGCTGCT ⁺ GTTGCCGTACCGTCAATT ⁺ TACAGACTTAATCCAGGGC ⁺ CAGGAAGATAGTTCTGTATGCC	600
83	G S D D E D A S V A E A A V A A T V N Y T D L I Q G Q E D S S D A	
	CACACATCTAATCAAACGA ⁺ ACCGCAATGGTGAGCACAAG ⁺ GATTCCCTTAATGGGGAAG ⁺ AGCCATAACCCCATCAA ⁺ TGAAGGAGTCAAGCCAAACACCT	700
116	H T S N Q T N A N G E H K D S L N G E R A I T P S N E G V K P N T S	
	NCT11	
	CCCTGGAGGGAATGACGTCT ⁺ TACCAATGGAATCTACACAGCAATCTAAAACGACATG ⁺ CTCATACATTGGCAGAACAC ⁺ GATCGTGGGCCAGAACACCA	800
150	L E G M T S S P M E S T Q Q S K N D M L I P L A E H D R G P E H Q	
	ACAGGATGATGAAGACAATG ⁺ ATGACGCTGACATTGATCT ⁺ CAAAAGGATATAAGCATGC ⁺ AGCCGGGTCTGCTGGAAGA ⁺ AAACCTACTACTTTGGCCACA	900
183	Q D D E D N D D A D I D L K K D I S M Q P G R R G R K P T T L A T	
	ACAGACGAGTGGAAAAGCA ⁺ AAAGAAAGACTCC ⁺ CATAAAG ⁺ AGTCGAAAGCGTCCGCCG ⁺ GAAACATCAACACTGCAAT ⁺ CAACGTTTAAAGCGACCTCC	1000
216	T D E W K K Q R K D S H K E V E R R R R R E N I N T A I N V L S D L L	
	^{Ssp I} TGCCCGTGAGAGAATCAAG ⁺ TAAAGCAGCAATAT ⁺ TGGCGCGTGGCGCTGAATACATTCAA ⁺ AAATGAAAGAAACCGATGA ⁺ AGCAACATCGAAAAGTGGAC	1100
250	P V R E S S K A A I L A R A A E Y I Q K L K E T D E A N I E K W T	
	GTTACAAAAATTGCTTAGCG ⁺ AGCAAAACGCATCGCAATT ⁺ AGCCAGTGCAAATGAGAACT ⁺ GCAGGAAGAACTGGGAAAT ⁺ GCTTACAAAGAAATCGAGTAC	1200
283	L Q K L L S E Q N A S Q L A S A N E K L Q E E L G N A Y K E I E Y	
	ATGAAACGCGTTTAAAGGA ⁺ AGGAGGGAATAGAATACGAGGATATGCACACTCACAAGAA ⁺ CAAGAGAACGAAAGAAAA ⁺ GCCTAGGAGCGATAATCCAC	1300
316	M K R V L R K E G I E Y E D M H T H K K Q E N E R K S T R S D N P H	
	ATGAGGCTTGAGAGTTAA ⁺ ATAGATAGCTAAATGTATT ⁺ CGAGTCTCCCTATGTATCT ⁺ CGCTCTCTGCCCCATAGCAC ⁺ CTCTGTTAAGTTTTATCAAT	1400
350	E A *	
	ATT	1403

Figure 1. DNA sequence of the gene encoding CP1. The DNA sequence is shown along with the protein translation of the 39-kDa open reading frame. The sequences of the NCT14 and NCT11 peptides are underlined. The *Ssp*I sites used to construct the *CEP1* gene disruption and the *Hind*III site used for the *tac* promoter fusion are overlined.

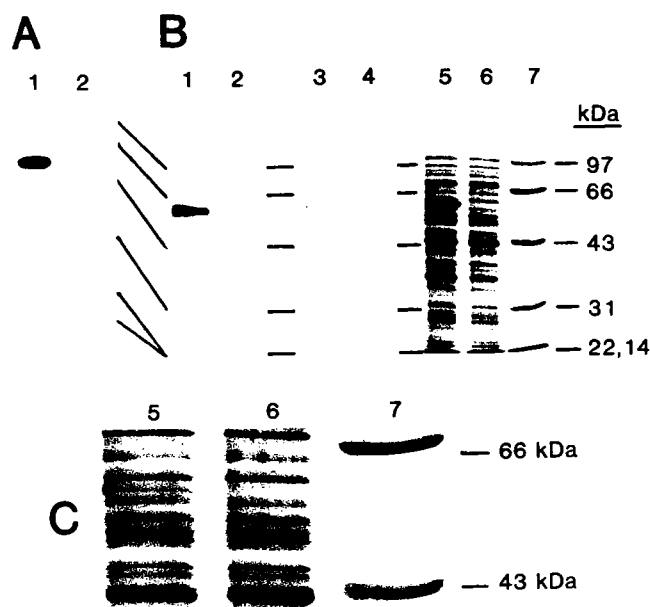


Figure 2. Expression of CP1 in *E. coli*. Proteins from bacteria containing the tac-CP1 plasmid pRB89-1 (CP1 open reading frame in the sense orientation) or pRB89-3 (CP1 open reading frame in the antisense orientation) were analyzed for CDEI binding activity by Southwestern blotting as described in Materials and Methods. (A) Proteins from a pRB89-1-bearing strain induced (lane 1) or uninduced (lane 2) by IPTG were analyzed on a 12% polyacrylamide resolving gel. (B) Proteins from IPTG-induced strains bearing pRB89-1 (lanes 1, 3, and 5) or pRB89-3 (lanes 2, 4, and 6) were analyzed on a 10% gel. After blotting, the membrane was cut, and one half was incubated with probe in the presence of a 25-fold excess of unlabelled CDEI DNA (lanes 3 and 4) or an equivalent amount of poly[d(IC)] (lanes 1 and 2). Lanes 5 to 7 show the results of Coomassie blue staining. Lane 7 contains protein molecular weight standards (BioRad). (C) Enlargement of the 40 to 70-kDa region of the Coomassie-stained gel. The putative CP1 translation product is indicated by dots (panels B and C, lanes 5).

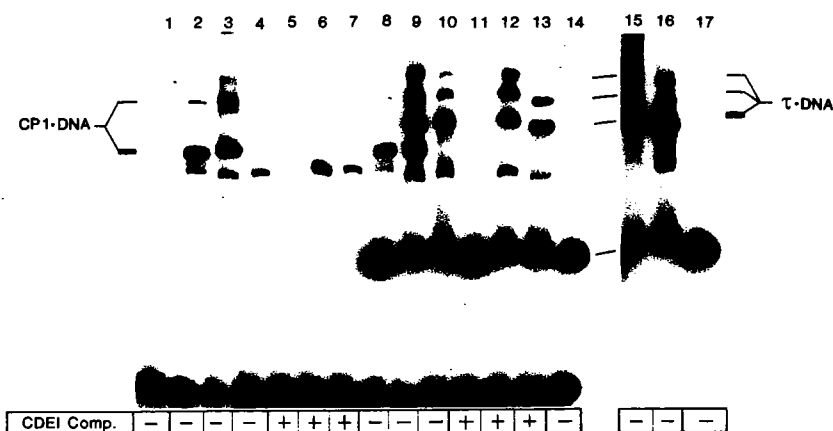


Figure 3. CDEI binding activity in *CEP1*⁺ and *cep1::URA3* strains. Crude whole cell extracts of the *CEP1*⁺ strain R11-4C (lanes 3, 6, 9, 12, 15) and the *cep1::URA3* strain R11-4B (lanes 4, 7, 10, 13, 16) were prepared and assayed for specific DNA-binding activity by gel shift assay as described in Materials and Methods. The reaction mixes in lanes 2, 5, 8 and 11 contain purified CP1 (5). The labelled probes were: CDEI only, lanes 1 to 7; both CDEI and tDNA, lanes 8 to 14; tDNA only, lanes 15 to 17. Where indicated below the figure, an approximate 500-fold excess of unlabelled CDEI competitor (comp.) DNA was added to the binding reaction mixes. For the crude extract assays, equal amounts of protein (25 μ g) were used per reaction. Lanes 1, 14, and 17 contain no added protein.